GENETICS

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THE INTERACTION OF MUTANTS AFFECTING VENATION IN DROSOPHILA MELANOGASTER. II. ADDITIVE AND PATTERN EFFECTS OF COMBINATIONS OF HAIRLESS, ENGRAILED AND CUBITUS INTERRUPTUS[1]

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THE interactions of the Hairless, engrailed and cubitus interruptus mutants relative to their interruption effects on the fourth longitudinal vein (L_4) in *Drosophila melanogaster* have been described in an earlier communication (HOUSE 1953). In the above study the effects of these mutants and their combinations were measured relative to certain rough grades of interruption of the fourth vein. The results of that study suggested an additive interpretation of apparent superadditive interactions if subthreshold components of mutant action were invoked. The present study is concerned with a quantitative evaluation of the additive hypothesis, and with a consideration of the nature of the regional pattern of fourth-vein interruption characteristic of the mutants and their combinations

MATERIALS AND METHODS

The following mutants were employed in the present study: cubitus interruptus (*ci*), engrailed (*en*), Hairless (*H*), Minute-4 (*M-4*) and eyeless-Russian (ey^R). Hairless (third chromosome, homozygous lethal dominant), engrailed (second chromosome) and cubitus interruptus (fourth chromosome) produce among other effects an interruption of the fourth longitudinal vein between the posterior crossvein and the margin of the wing. The interactions of these mutants relative to this trait is the subject of the present report. For a fuller description of the effects of these mutants alone and in combination reference hould be made to the first paper in this series (HOUSE 1953). Eyeless-Russian (fourth chromosome), producing a variable reduction in the size of the eye and having no modifying effect on L_4 interruption (STERN 1943; HOUSE 1953), was used as a marker for the fourth chromosome. Minute-4 (fourth chromosome), which constitutes a deficiency for the *ci* locus but not for that of ey^R, was used to produce hemizygosity of the *ci* and ci^+ alleles.

The genotypes compared and the crosses from which they segregated are as follows: (1) *ci/ci* (2) *H/+; ci/ci* (*ci/ci* × *H/+; ci/ci*) (3) *en/en* (4) *en/en; H/+* (*en/en* × *en/en; H/+*) (5) *H/+* (*+/+* × *H/+*) (6) *en/en; ci/ci* (7) *en/en; H/+; ci/ci* (*en/en; ci/ci* × *en/en; H/+; ci/ci*) (8) *ci* ey^R

[1] Adapted in part from a thesis submitted to the Graduate Division of the University of California at Berkeley in partial satisfaction of the requirements for the degree of Doctor of Philosophy

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/ci ey^R (9) *ci ey^R/M-4* (10) *H/+; ci ey^R/ci ey^R* (11) *H/+; ci ey^R/M-4* (*ci ey^R/M-4* × *H/+; ci ey^R/ci ey^R*) (12) *en/en* (13) *en/en; M-4/+* (*en/en* × *en/en; M-4/+*) (14) *en/en; ci ey^R/++* (*en/+; ci ey^R/++* × *en/en; ci ey^R /ci ey^R*). All crosses involve single pair matings at 26 ± 0.5°C in half-pint milk bottles, utilizing the standard cornmeal-molasses-agar food medium enriched with yeast. The flies on which the measurements to be described below were made constitute a random sample of those emerging from each cross over an eight-day period of collection, the parent females in each case having been permitted to lay eggs for seven to eight days prior to removal.

Wings from a random sample of each genotype under investigation were dissected off and mounted in euparol. By means of a camera lucida these wings were projected at desk height, and the length of L_4 from the posterior crossvein to the wing margin was divided into twenty equal intervals. The individual intervals were then scored for the presence or absence of vein material, vein being considered to be present if the vein segment in any interval was equal to or exceeded one-half of the length of the interval. The subdivision into twenty equal intervals of variable lengths of L_4 was accomplished by the use of a grid ruled with twenty-one equally spaced parallel lines. For any particular length of L_4 this grid was oriented on the table top in such a manner that the points of intersection of L_4 with the posterior crossvein and wing margin corresponded with the points of intersection of L_4 with the first and twenty-first lines of the grid, respectively. The equally spaced parallel lines of the grid thus divided the varying lengths of the fourth vein into twenty intervals of equal length. This method of measurement furnishes data concerning the total amount of vein material present in a given wing measured to the nearest 5 percent, as well as providing a basis for determining the frequency of venation within given regions of the fourth vein.

RESULTS

Additive interaction of en, ci, and H

In figure 1 are presented frequency diagrams for females of the *ci/ci, en/en, H/+, en/en; ci/ci, H/+; ci/ci, en/en; H/+,* and *en/en; H/+; ci/ci* genotypes. As seen from these curves, *en/en; H/+* is the only genotype whose distribution does not overlap either the 0% or 100% levels. The remainder of the distributions either show incomplete penetrance, a certain percentage of flies having completely normal L_4 venation (*en/en, H/+, ci/ci*), or else they intersect the 0% line, a certain proportion of the sample measured showing a complete absence of L_4 distal to the posterior crossvein. In terms of a threshold interpretation of penetrance and expressivity attributable chiefly to GOLDSCHMIDT (1938 (review), 1945, 1951) we may interpret these interrupted distributions in the following manner.

Let us assume that the *en, ci,* and *H* loci are in some manner involved in the elaboration of a substance P which is concerned in some way with vein formation. Furthermore, let us assume that P is related to L_4 venation in such a way that an increase in the amount of P leads to increased venation except where

this increase is masked by the operation of thresholds. When the average concconcentration of P in an individual wing reaches a lower threshold of minimal response (0% level of L_4 venation), vein material starts to appear in the region distal to the posterior crossvein. With increasing concentrations of P above this level of minimal response, higher percentage levels of L_4 venation result. When P reaches the concentration level of the upper threshold of maximal response (100% L_4 venation), L_4 becomes complete, and any further increase in the concentration of P above this point will result in no measurable effect.

The assumption that increases in P, within the range between the two thresholds, are reflected by increases in the percentage of L_4 present does not necessarily imply a linear relationship between concentration of P and percentage of vein manifestation. For our present purposes we shall assume merely that changes in P are reflected rather accurately by changes on the percentage scale, and shall employ this scale as our metric, both in the range of measurable

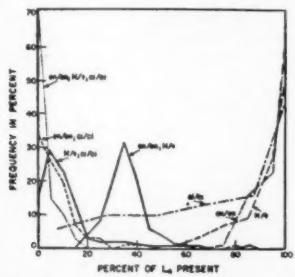

FIGURE 1.—Frequency distributions for the degree of L_4 interruption in females of *en/en*, *ci/ci*, *H/+* and their combinations.

change and in the regions extending beyond the lower and upper thresholds. With respect to a population of individuals of a given genotype, the penetrance of L_4 interruption reflects the portion of the curve of variation for the genotype which extends below the upper threshold of maximal response. Average expressivity for the genotype becomes a measure of the distance below or above this threshold occupied by the population mean. A direct determination of the mean and variance of the curve of variation of a given genotype is possible only when the distribution occupies the range between the lower and upper thresholds (0–100%), as is the case for *en/en; H/+*. For the estimation of means and variances of those genotypes showing partial overlap of either the upper or lower thresholds, indirect methods must be utilized.

A normal frequency curve may be represented in either one of three ways, depending upon its representation along the vertical axis. If frequency is plotted along the vertical axis for each value of the variate along the abscissa, the usual form of the curve is obtained. If areas of the normal curve are accumulated from one end, the integrated normal sigmoid curve results. This sigmoid curve may be transformed into a straight line by representing along the y-axis the

normal deviates corresponding to any given cumulated frequency (WRIGHT 1926). This is possible since for the normal distribution there is a fixed relationship between the frequency accumulated from a given end up to a given point and the deviation of that point from the mean in terms of standard deviations. This normal deviate has been designated the "normal equivalent deviate." Where a value of 5 is added to each normal deviate to avoid the use of negative quantities, the resulting measure has been labeled the probit (BLISS 1935). Since the third type of representation is essentially a plot of normal deviate against normal deviate, the sigmoid accumulated frequency curve is made linear. This latter representation furnishes a graphical basis for estimation of the mean and standard deviation of a set of measurements where such a set approximates a normal distribution. For each value of the variate along the x-axis, the normal equivalent deviate is plotted in place of the cumulated frequency up to that point. An estimate of the mean is given by the intersection

TABLE 1

Means and variances of the mean for en/en, ci/ci, H/+ and their combinations; and for three estimates of +/+.

Genotype	♂♂			♀♀		
	n	$\bar{x}$	$\sigma_{\bar{x}}^2$	n	$\bar{x}$	$\sigma_{\bar{x}}^2$
H/+	220	111.8	10.70	152	98.6	3.75
en/en	102	101.1	1.79	116	99.8	1.81
ci/ci	122	75.7	16.94	193	87.2	13.49
en/en; H/+	100	34.2	0.95	132	35.6	0.49
en/en; ci/ci	98	6.8	2.15	99	8.1	2.92
H/+; ci/ci	100	12.8	3.65	100	13.4	3.13
+/+ (*en; ci*)	....	170.0	20.9	....	178.9	18.2
+/+ (*H; ci*)	....	174.7	31.3	....	172.4	20.37
+/+ (*en; H*)	....	178.75	13.4	....	162.8	6.05

of the fitted straight line and the line with the normal equivalent deviate value of zero. An estimate of σ is given by the distance along the x-axis cut off by the intersection with this axis of the perpendiculars dropped from the points of intersection of the lines of normal equivalent deviate values of 0 and -1.0 with the fitted straight line. The graphical method can be made applicable to the present data by determining the linear relationship expected in the normal deviate plot from that portion of the curve of variation occupying the region between the two thresholds.

An alternative method to the graphical estimation of the mean and standard deviation of truncated normal distributions is that given by IPSEN (1949). In the latter method tables are provided for the determination of the true mean and standard deviation of the assumed interrupted normal curves from the ratio of the variance of the given truncated distribution to its mean. Estimates of the means and standard deviations of the supposed normal curves equivalent to the interrupted distributions of figure 1 for the *en/en*, *H/+* and *ci/ci* genotypes were computed by both graphical and tabular methods. The estimates by

the two methods were in close agreement. The means for *en/en, H/+* and *ci/ci* estimated by the method of IPSEN are given in table 1.

Figure 1 indicates that the distributions for *en/en; ci/ci* and *H/+; ci/ci* are considerably skewed to the right, showing a great enough departure from normality to militate against the use of the normal equivalent deviate for estimates of means and standard deviations. To correct for the too high mean values obtained if the actual distributions are used, the distributions have been extended to the left of the lower threshold line by assuming an essentially symmetrical distribution. The resulting curves for *en/en; ci/ci* males and females are indicated in figure 2. Such curves were used for calculating by ordinary methods the values of $\bar{x}$ and $\sigma_{\bar{x}}^2$ for *en/en; ci/ci* and *H/+; ci/ci* given in table 1

In order to test statistically the hypothesis of additive action of *en, H* and *ci* in combination it becomes necessary to know the sampling variances of the estimated means given in table 1 for the interrupted *H, en* and *ci* distributions. The estimate, $\sigma_{\bar{x}}^2 = \sigma^2/n$, will be too low since this method does not take into

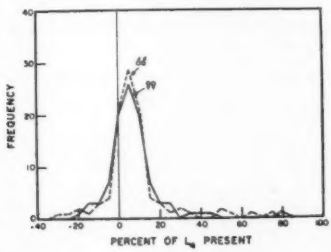

FIGURE 2.—Reconstruction of a theoretical frequency curve for *en/en; ci/ci* males and females. (Further description in text.)

account the relative weight that should be given to the mean of a truncated distribution in comparison to the mean of a corresponding complete distribution of the same n and σ. Nor does the method take into account the increase in the variance of the estimated standard deviation of a truncated distribution with increasing degree of truncation. IPSEN (1949) has provided formulae and tabular data for estimating the sampling variance of the true mean of a truncated distribution in terms of the weighing factor of the mean and the sampling variance of the estimated standard deviation. These formulae and tables have been used to calculate the variances of the means of the *en/en, H/+* and *ci/ci* genotypes given in table 1.

The means in table 1 have been plotted in figure 3 for both males and females. The distributions about these means have been indicated by normal curves based on the respective estimated standard deviations. With reference to the interpretation given above to the truncated distributions of figure 1 it is obvious that the measurement of mutant action should not be relative to the threshold itself but to some theoretical value of P lying above the upper threshold in the wild-type fly. Estimates of this wild-type P level, based on the

assumption of an additive interaction of the mutants in the combination genotypes, may be obtained from the means of the distributions of figure 3.

If we represent by $\bar{x}$ the average level of P in the wild-type fly, by $\bar{x}_{ab}$ the mean of a combination genotype involving two mutant substitutions, and by $\bar{x}_a$ and $\bar{x}_b$ the means of the two single-mutant genotypes of which the combination is composed, then the following relationship can be shown to be true: $\bar{x}_+ = \bar{x}_a + \bar{x}_b - \bar{x}_{ab}$. We may accordingly make use of the *en/en; H/+*, *en/en; ci/ci* and *H/+; ci/ci* means to arrive at three independent estimates of the

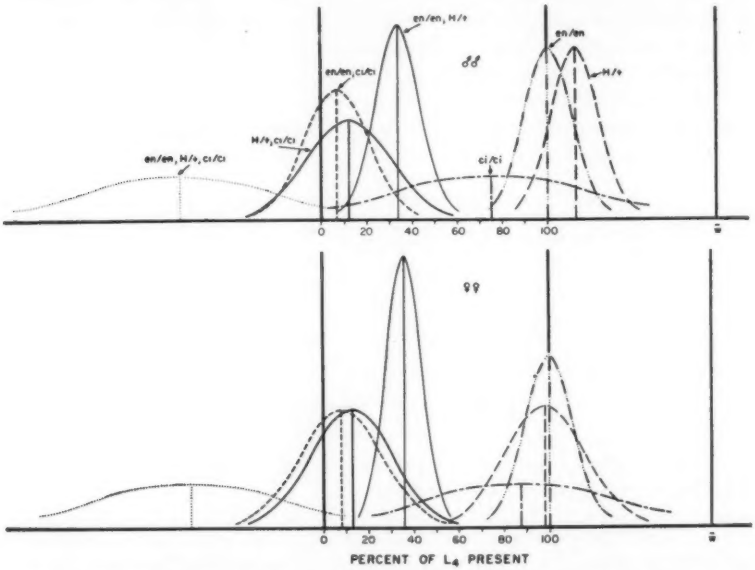

FIGURE 3.—The cumulative effects of *en/en*, *ci/ci* and *H/+* in combination, assuming subthreshold effects and additive action. (Further description in text.)

wild-type mean (table 1). These three estimates should show agreement with one another within the limits of sampling error if the assumption of additive action is correct.

The mean, $\bar{w}$, of the three estimates based on the three two-mutant combinations, *en/en; H/+*, *H/+; ci/ci* and *en/en; ci/ci* is given by the following expression: $\bar{w} = [2(\bar{x}_a + \bar{x}_b + \bar{x}_c) - (\bar{x}_{ab} + \bar{x}_{ac} + \bar{x}_{bc})]/3$. From this expression and the earlier one for $\bar{x}_+$ can be derived an expression for the difference, d, between $\bar{w}$ and any two-mutant estimate of $\bar{x}_+$. This difference and its sampling variance are given by the following expressions:

$$d = \bar{w} - \bar{x}_{+(ab)} = (\bar{x}_a + \bar{x}_b - 2\bar{x}_c + \bar{x}_{ac} + \bar{x}_{bc} - 2\bar{x}_{ab})/3$$

$$\sigma_d^2 = (\sigma_{\bar{x}_a}^2 + \sigma_{\bar{x}_b}^2 + 4\sigma_{\bar{x}_c}^2 + \sigma_{\bar{x}_{ac}}^2 + \sigma_{\bar{x}_{bc}}^2 + 4\sigma_{\bar{x}_{ab}}^2)/9$$

It may be assumed that the true wild-type mean is given by $\bar{w}$, the average of the three two-mutant estimates. Our null hypothesis is then that the deviation of each two-mutant estimate from $\bar{w}$ is due entirely to sampling error. The test of significance therefore takes the form of a c test, expressing the ratio of the actual difference between a two-mutant estimate and $\bar{w}$ to the standard error of that difference. Such a test in males for each of the three estimates of the wild-type mean gives a probability figure greater than 5%. In females, however, the deviations of $\bar{x}_+$ from $\bar{w}$ are significant at the 1% level for two of the three wild-type estimates.

It must be concluded, therefore, that relative to the conditions under which the interaction study was conducted, a strictly additive action in females of the *en, ci* and *H* mutants in combination is ruled out. It should be emphasized, however, that the described interaction studies were not conducted with isogenic stocks, and that some of the genotypes compared conceivably possess entirely different levels of modifier action. The departures from an additive picture are possibly the results of differential modifier action in the different genotypes and might not in reality result from non-additive interaction of the main mutant substitutions.

For the purpose of completing the picture of cumulative interaction of *en, ci* and *H*, the mean for *en/en; H/+; ci/ci* has been included in figure 3. The location of this genotype is based on the assumption of an additive interaction of *en, ci* and *H* relative to the wild-type level $\bar{w}$. That the actual *en/en; H/+; ci/ci* mean may lie closer to the lower threshold than figured is suggested by a second estimate based on the normal equivalent deviate (relative to the lower threshold) of the *en/en; H/+; ci/ci* distribution (♂♂, 0.81; ♀♀, 0.58) together with the assumption that $\sigma_{en;\,H,\,ci} = \sigma_{ci}$. The resulting calculations yield a value of −35% for males and −25% for females.

The regional pattern of interruption of the fourth vein

In the considerations so far, concern has been chiefly with the total amount of L_4 interruption present in a given mutant or combination of mutants, without regard to the distribution of that interruption. This emphasis has tended to obscure the pattern differences that exist among the various genotypes. The nature of the patterns will now be examined in some detail.

Within a given segment of L_4, it appears that the vein-forming reaction, aside from a thinning phenomenon, is a quantal, or all-or-none, reaction. We may characterize the venation response within any segment for any given genotype by the percentage of individuals in a population representative of that genotype that shows vein material within the segment in question. Determinations of this type for different intervals along the course of L_4 make it possible to characterize a genotype by means of a frequency profile measuring the intensity of the vein-forming reaction along the length of L_4. These frequency profiles are readily calculated from a series of measurements of the type described earlier, where the amount of L_4 in individual wings was determined to the nearest 5%. From the same series of measurements it is possible

also to determine the frequency of venation for a given genotype at each of the 20 intervals of L_4. The all-or-none nature of the venation reaction in any segment of L_4, however, suggests that to measure the vein-forming potentialities along L_4 a series of transformed frequencies should be used rather than the actual frequencies of venation.

Two basic assumptions underlie the proposed transformation of venation frequency: first, that for any given genotype and interval of L_4 there exists a normal distribution of the theoretical vein-forming substance, P; and, second, that for the vein-forming action to proceed at this interval, the concentration of P must exceed a minimal threshold value, $\overline{P}_T$. All those individuals whose P values exceed $\overline{P}_T$ will show vein in the segment in question; all those whose P values fall below $\overline{P}_T$ will show no vein at this interval. The percentage, or frequency, of venation within the interval is measured by the area under the normal curve exceeding $\overline{P}_T$. The intersection of $\overline{P}_T$ with the frequency distribution of P can be determined relative to the mean, $\overline{P}$, by transforming the

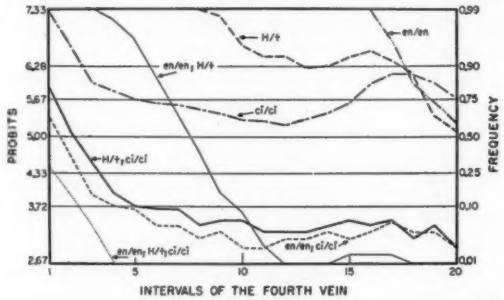

FIGURE 4.—The regional pattern of interaction in females of *en/en*, *ci/ci*, *H/+* and their combinations.

frequency of venation within any segment to a "normal equivalent deviate." That is, each frequency corresponds to a normal deviate expressed as a difference between the two concentrations, $\overline{P}$ and $\overline{P}_T$. Since the frequency of venation, F, is a probability function of the normal deviate, $(\overline{P}-\overline{P}_T)/\sigma$, the latter quantity may be considered as the inverse probability function of the frequency (WRIGHT 1920). The inverse probability transformation is of course the "normal equivalent deviate," or probit, transformation already mentioned.

Transformed frequency profiles have been plotted for the various genotypes investigated in figures 4 through 6. The twenty regions of the fourth vein, numbered from the posterior crossvein to the wing margin, are represented along the x-axis. On the vertical axis have been plotted the transformed frequencies or probits for each interval and genotype. For convenience, the frequencies corresponding to various probit values are indicated along the vertical scale to the right.

As we have seen, the probit transformation affords a measure of the difference between two concentrations, $\overline{P}$ and $\overline{P}_T$. A change in the relationship of

$\overline{P}$ and $\overline{P}_T$ could be effected by a change in $\overline{P}$, in $\overline{P}_T$, or conceivably in both. However, for the purpose of comparison of the profiles of figures 4 through 6 we shall assume a fixed threshold, $\overline{P}_T$, located at the 50 percent level (probit = 5), and interpret the various profile points as changes in the mean concentration of $\overline{P}$.

In figure 4 are presented the profiles for *en/en, ci/ci, H/+* and their combinations, based on the same samples of wings constituting the distributions of figure 1.

Interruption of L_4 for cubitus interruptus is at a maximum in the central regions of the fourth vein, and at a minimum in the region just distal to the posterior crossvein. In addition, both males and females show an increase in venation in the approximate region of intervals 16 through 18, with a drop in frequency at the distal tip of L_4. A χ^2 comparison of the frequency of venation at each of the following pairs of intervals shows a significant difference at the one percent level for both males and females: 3,12; 12,17; 17,20.

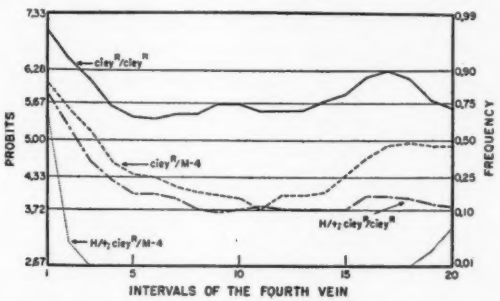

FIGURE 5.—The regional effect in females of the increase in dosage of the *ci* allele in the presence and absence of the Hairless substitution.

The strictly terminal interruption characteristic of engrailed, and the partly terminal, partly interstitial interruption of Hairless are reflected in the profile plots for these genotypes. In the former mutant only the most distal fourth of L_4 shows interruption. Hairless shows a variable interruption over one-half of the fourth vein, with maximal vein interruption in the terminal region.

The profiles for the combination genotypes show in general a cumulative interaction at each interval of L_4, with suggestions of subthreshold effects of *en, ci* and *H* in most segments. For example, the combinations *en/en; H/+, en/en; ci/ci* and *H/+; ci/ci* all show decreased frequencies of venation in intervals unaffected by one of the contributing mutants, and in *en/en; H/+*, segments unaffected by either mutant singly show interruption. The implication of these facts in terms of subthreshold interpretation is that the wild-type profile lies not at the 100% level, but somewhere above, and that $\overline{P}$ must therefore show a considerable decrease in concentration for most regions of L_4 before interruption of the vein occurs.

In figure 5 are plotted the probit profiles for *ci ey^R/ci ey^R, ci ey^R/M-4,*

H/+; ci ey^R/ci ey^R and *H/+; ci ey^R/M-4*. Comparison of *ci ey^R/ci ey^R* with *ci ey^R/M-4* shows an increase in normality of venation for each interval of L_4 as the *ci* allele is increased from one to two doses. The increment toward normality, however, varies considerably among the intervals, being at a minimum in the proximal and distal regions and at a maximum in the central portions of L_4. For example, interval 11 in females shows an increase in venation frequency from 9.6% in *ci ey^R/M-4* to 70.0% in *ci ey^R/ci ey^R*, whereas interval 20 exhibits an increase only from 46.5% to 72.5%. Comparison of *H/+; ci ey^R/M-4* and *H/+; ci ey^R/ci ey^R* indicates a similar increase in normality of venation accompanying increased *ci* dosage in the presence of the Hairless substitution.

The transformed frequency profiles for the *en/en, en/en; M-4/+* and *en/en; ci ey^R/++* genotypes are presented in figure 6. Comparison of *en/en; M-4/+* and *en/en* permits evaluation of the effect of an increase from one to two doses of the normal *ci* allele (*ci$^+$*) in the presence of the engrailed substitution.

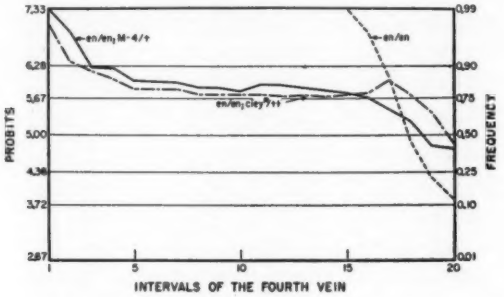

FIGURE 6.—The regional effect in females of changes in dosage and allelic combination at the *ci* locus in the presence of the engrailed substitution.

Plainly, the proximal and central intervals of L_4 respond differently to this increase in *ci$^+$* dosage than do the most distal intervals. In the latter region for females there is a decrease in venation with increase in *ci$^+$* dosage (e.g., interval 19, $\chi^2 = 30.62$, d.f. = 1, $p < 0.01$) in contrast to the increase in the frequency of venation for the remaining intervals. For males an increase from one to two doses of *ci$^+$* in the presence of engrailed brings about an increase in normality of L_4 venation at all intervals except the most proximal and distal.

The comparison *en/en; M-4/+* vs. *en/en; ci ey^R/++* tests the effect of adding the *ci* allele to one dose of the *ci$^+$* allele in the presence of engrailed. For most of the intervals along L_4 there is no significant effect on venation for females of the *ci* addition, although for all intervals up to 16 *en/en; M-4/+* shows consistently a higher frequency of venation. For intervals 17, 18 and 19, however, there is a reverse of the situation, *en/en; ci ey^R/++* exhibiting a significantly greater venation than *en/en; M-4/+*. The comparison, *en/en; M-4/+* vs. *en/en; ci ey^R/++*, in males presents essentially the same picture as in females. All intervals up to 17 show a consistently higher frequency of venation for

en/en; M-4/+, with the difference reaching the 5% level of significance in various regions. For intervals 17, 18, 19 and 20 *en/en; ci ey^R/++* becomes more normal, the difference being significant at the one percent level for intervals 18 and 19.

The general resemblance of the *en/en; M-4/+* and *en/en; ci ey^R/++* profiles to those for *ci/ci* and *ci ey^R/ci ey^R* should be noted. This is to be expected if heterozygous *ci* and hemizygous *ci$^+$* produce a cubitus interruptus effect, but at a subthreshold level, such an action being brought into the range of measurable effect through cumulative interaction with engrailed.

Correlation between different regions of the fourth vein

The preceding analysis points to a difference in the localized conditions at various points along L_4 under which the three mutant substitutions manifest themselves. Such facts alone, however, furnish no basis for conclusions as to the nature of the correlating mechanisms responsible for the various patterns of interruption. To proceed with this sort of analysis, it is first necessary to determine the correlation respecting the presence of venation between different parts of the fourth vein, and between homologous segments of L_4 in right and left wings.

Such correlation coefficients were calculated by means of YULE's theoretical correlation coefficient for the *en/en* (intervals 17, 18, 19), *ci/ci* (intervals 2, 5, 8, 11, 14, 17, 20), and *H/+* (14, 16, 18, 20) genotypes. The formula is,

$$r = \frac{ad - bc}{\sqrt{(a + c)(b + d)(a + b)(c + d)}}$$

where a represents the number of individuals showing vein material in both intervals under consideration, d the number showing interruption in both intervals, b the number showing vein material for one interval and interruption for the other, and c the reverse of the latter situation. The calculation takes the form of a fourfold table. The significance of the correlation coefficient may be determined by comparing actual values of a, b, c and d with those expected if the occurrence of the various combinations were determined on a chance basis only (zero correlation). The test of significance is, therefore, a χ^2 test with one degree of freedom. Wherever expected numbers have been 5 or less YATES' correction has been applied.

The results of the above correlation calculations are presented in tables 2 and 3. Table 2 presents the intra-vein and right-left correlation coefficients for a *ci/ci* stock inbred by brother-sister pair matings for 12 generations prior to the correlation determinations.

Independence of developmental processes on the right and left sides of the body has in general been taken for granted by Drosophila workers, although data to the contrary have been reported for certain mutants (MARGOLIS 1936). Assuming such independence for the vein-forming reaction, and in the absence of sources of variation acting on the individual as a whole (e.g., segregation of genetic modifiers and variation in factors of the external environment such

TABLE 2

Intra-vein and right-left correlations between intervals of the fourth vein for an inbred ci stock.

	Right-left correlation		Correlation between intervals of left wing[1]						
			2	5	8	11	14	17	20
♀♀[2]	0.08 #†	2	1.00	0.54	0.32	0.38	0.29	0.24	0.18
	0.07 #	5		1.00	0.66	0.51	0.40	0.35	0.23
	0.20 **	8			1.00	0.68	0.46	0.31	0.28
	0.11 #	11				1.00	0.62	0.27	0.24
	0.11 #	14					1.00	0.43	0.43
	0.04 #	17						1.00	0.66
	0.01 #	20							1.00
♂♂[2]	0.04 #	2	1.00	0.54	0.36	0.34	0.44	0.38	0.31
	0.16 *	5		1.00	0.67	0.55	0.48	0.31	0.29
	0.06 #	8			1.00	0.65	0.48	0.27	0.35
	0.02 #	11				1.00	0.67	0.26	0.23
	0.02 #	14					1.00	0.38	0.30
	0.15 *	17						1.00	0.48
	0.07 #	20							1.00

[1] All intra-vein correlations significant at 1 percent level.
[2] Right-left and intra-vein correlations based on 200 individuals each.
† Expected numbers in two-fold table < 5, Yates' correction applied.
# Probability > .05.
* Probability < .05.
** Probability < .01.

TABLE 3

Intra-vein and right-left correlations between intervals of the fourth vein for H/+ and en/en[1].

	H/+[2]				*en/en*[3]				
Right-left correlation	Correlation between intervals of left wing								Right-left correlation
	14	16	18	20	20	19	18	17	
0.02 *†	1.00	0.44 **†	0.12 #†	0.15 *	0.14 #†	0.21 *†	0.38 **	1.00	0.15 #†
0.20 **†		1.00	0.39 **†	0.23 **	0.37 **	0.54 **	1.00		0.46 **
0.16 **			1.00	0.46 **	0.68 **†	1.00			0.41 **†
0.40 **				1.00	1.00				0.48 **†

[1] *en/en* Flies from the cross, *en/en*; *M-4/+* × *en/en*.
[2] Right-left and intra-vein correlations based on 265 individuals each.
[3] Right-left correlations based on 106 individuals; intra-vein correlations based on 154 individuals.
† Expected numbers in two-fold table < 5, Yates' correction applied.
# Probability > .05.
* Probability < .05.
** Probability < .01.

as temperature), the correlation between right and left sides should be zero. With residual heterozygosity, or large fluctuations in temperature, deviation from zero right-left correlation would be expected. On the other hand, correlations between different regions of L_4 on the same side would be zero only if variations in the conditions of the internal environment affecting the venation in a given region produced effects limited to the immediate vicinity of that

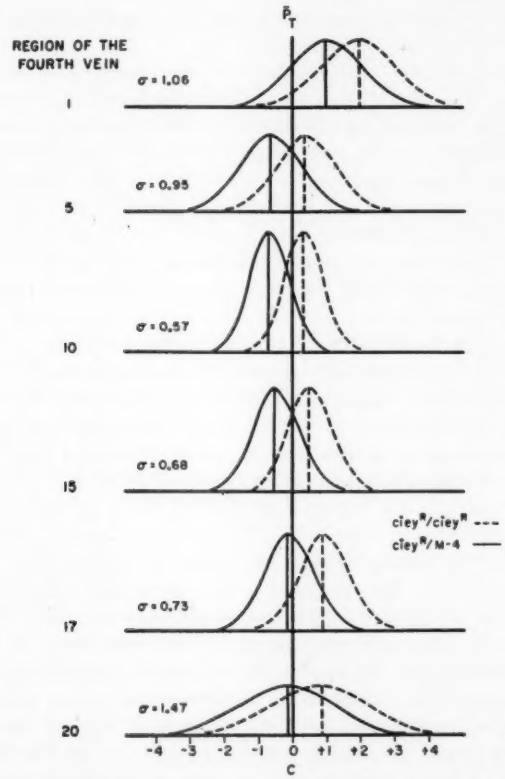

FIGURE 7.—Theoretical distributions of P in *ci ey*^{R}/*ci ey*^{R} and *ci ey*^{R}/*M-4* for six regions of the fourth vein. (Further description in text.)

region, and if genetic and external environmental homogeneity obtained. It follows that the intra-vein correlations for any genotype must be considered with reference to the degree of right-left correlation found for the genotype as a base level, since the factors operating to produce positive right-left correlation also bring about correlation between intervals of the same wing. Significant correlations between two vein intervals of one side may only be attributed to the covariance of common factors of the internal environment which affect

the venation response in the case of those stocks reduced to genetic and environmental (external) homogeneity (zero right-left correlation).

In general, the results of tables 2 and 3 show a higher correlation between intervals of the same vein than between homologous intervals of the right and left veins, the intra-vein correlation decreasing with distance and approaching the level of right-left correlation for the regions most distantly separated. The significant right-left correlations for most intervals in the *H*/+ and *en/en* genotypes of table 3 suggest a residual heterozygosity for modifiers affecting the venation response in these mutants.

The inbred *ci/ci* stock, on the other hand, shows absence of right-left correlation for most intervals, an indication that inbreeding has led to the development of a stock homozygous for modifiers affecting the venation response. However, in the homozygous *ci/ci* stock there is still high correlation between neighboring L_4 intervals, although the correlations fall off with increasing distance between the regions compared, and approach the level of right-left correlation for the intervals most distantly separated. A similar picture of low right-left correlations and decrease of correlation with distance has been presented by CHASE (1939) with respect to presence or absence of pigmentation in the coats of guinea pigs.

It may be concluded that random variations in the factors of the internal environment affecting venation in any given L_4 region produce a similar effect in other regions of the same side to a degree inversely related to the distance separating the regions. For the most widely separated regions the correlated effects of local variations in internal environmental factors affecting venation is so small as to bring about near independent vein responses in these regions.

DISCUSSION

In the preceding analysis a theoretical substance, P, has been assumed to play some basic role in the vein-forming reaction in the Drosophila wing. As to the manner in which such a substance would be related to the actual formation of vein material we can only speculate. There are, however, certain data from Drosophila embryology that furnish a basis for speculation.

WADDINGTON (1940) has described the development of venation in the wild type and in certain mutants of *Drosophila melanogaster*. We may summarize those facts of normal development that are pertinent to this discussion as follows.

As the wing blade escapes from the peripodal membrane in the early prepupa the dorsal and ventral surfaces come together except along certain longitudinal lines where spaces are left. The channels left by this failure of the two surfaces to make contact constitute the first system of blood lacunae. The wing at this stage has the appearance of a somewhat cylindrical block of tissue, the two surfaces being composed of high cylindrical cells whose bases are drawn out into long protoplasmic cylinders. These protoplasmic bases meet in the midplane to obliterate the cavity of the wing fold except in the areas marking the prepupal system of veins. This system consists of five lacunae, four of

which are the prepupal representatives of the adult veins, L_1, L_3, L_4 and L_5. In the late prepupa the wing undergoes an expansion in which the cells of the two surfaces of the wing are drawn out into long thin processes that stretch from one surface of the wing to the other. During this expansion the prepupal system of veins becomes obscured. Following the expansion period, which extends into the early pupal stage, the wing contracts to a thin blade. During this contraction the epithelial cells of the two surfaces, except in the regions overlying and underlying the potential veins, put out basal processes which meet and fuse in the midplane to form a central membrane. The channels left due to the failure of the vein cells to put out basal processes constitute the system of veins found in the adult wing.

WADDINGTON has also described the development of several mutants producing local absences of veins. Among these are tilt (*tt*) and veinlet (*ve*), the former producing an interruption of L_3, the latter removing the distal portions of L_3, L_4 and L_5. The prepupal venation in both of these mutants is completely normal, as well as the pupal venation when first formed. Soon after the pupal veins appear, however, the vein sections affected in these mutants disappear, due to the protrusion of basal processes from vein cells nor normally forming them. WADDINGTON concluded from the above observations that the vein sections affected in veinlet and tilt at first behave like vein material and fail to put out processes, but later fail to retain their vein character and form basal processes just as the intervein cells do. He assumes that the vein cells have the capacity to put out processes, but that normally this capacity is inhibited. In tilt and veinlet this inhibition apparently breaks down over the section of vein affected. WADDINGTON has described the *ci* interruption as being present in the earliest stage at which the prepupal vein system can be distinguished. Presumably the absence of sections of L_4 in this stage in *ci*/*ci* are due to the extension of cytoplasmic bases by vein cells in the affected region, these bases fusing to obliterate the potential vein cavity.

On the basis of WADDINGTON'S observations it might be assumed that the presence or absence of vein material in any particular segment is a function of the absence or presence of cytoplasmic extensions or outgrowths from the epithelial cells overlying a particular section of potential vein lacuna. If in a particular region these processes are sent out by the overlying and underlying epithelial cells an intersurface meshwork of processes is formed (viz., in the normally non-vein areas), and there is no opportunity for the formation of a lacuna. However, in regions where these processes do not grow out, a space is left to constitute the vein lacuna, and around this the later differential chitinization takes place. Thus the vein-forming process in any given L_4 region would appear to be an all-or-none reaction, the outcome of which is contingent on the conditions controlling the presence or absence of outgrowth of cytoplasmic processes in the cells over- and under-lying the particular region in question. The normally present inhibition of outgrowth of processes along the vein channels could conceivably be mediated by way of an inhibitory substance identifiable with our theoretical material, P. Were P to fall below a critical

threshold value in a given L_4 interval, outgrowth of basal processes from the epithelial cells would take place, with resulting obliteration of vein lacuna. As long as the concentration of the inhibiting material remains above the threshold, cytoplasmic outgrowth from the overlying and underlying epithelial cells remains inhibited, resulting in the formation of normal vein channels.

While the speculative nature of the above interpretation of the nature of the vein-forming reaction must be stressed, the interpretation does provide a frame of reference for thinking about mutant action in embryological terms, such as to serve as a useful guide in continued studies of venation mutants.

The present data afford little basis for speculations concerning the nature of the primary gene action at the three loci investigated. With reference to the *ci* locus STERN (1943) has demonstrated that changes in quantity of the *ci* allele from one to two and from two to three doses lead to increasing normality of L_4 venation. STERN has assumed that *ci* and *ci*$^+$ react with a cellular substrate, S, producing a product, p, which is in some manner positively correlated through a subsequent chain of reactions ($p^1 \rightarrow p^2 \rightarrow p^3 \ldots\ldots$) with the appearance of venation. The fact that changes in quantity of the *ci* allele from one to two and from two to three doses lead to increasing normality of venation is interpreted as signifying an excess of S over the amount utilized by one or two *ci* alleles. The results of changes in dosage of the *ci* allele also imply that the *ci* allele acts toward normality of L_4 venation like the wild-type allele, but that *ci* does so less efficiently.

In a similar manner the increase in normality of venation in all regions of L_4 with increase in the number of *ci* alleles could be considered as evidence for an excess of S in each region of L_4 over that utilized by one *ci* allele. The lowered frequency of venation in central regions of the fourth vein as compared to terminal regions in *ci*/– and *ci*/*ci* can be attributed to higher thresholds for the venation response in the central regions. Assuming that there is no limitation of substrate in any region of the fourth vein relative to utilization by two *ci* alleles, and that the variance of the theoretical normal distributions of P in each region are identical, the change from one to two doses of *ci* should lead to equal increments of effect on the probit scale. The fact that this is not so may be due either to the fact that there is insufficient substrate for full utilization by two *ci* alleles in the terminal regions compared to no such limitation in the central regions; or to the fact that the accidents of development which operate to produce the variation in P about $\overline{P}$ are not equivalent in all regions. If the former condition were true, we would expect the damping of the effect of increased *ci* dosage in the terminal regions as compared to the central intervals that is shown by the data. With respect to the normal allele, *ci*$^+$, we would then have to suppose that the increased efficiency of this allele over that of *ci* (STERN 1943) makes possible the formation of sufficient P for normality in all regions in *ci*$^+$/– and *ci*$^+$/*ci*$^+$ by means of the more efficient utilization of whatever amount of substrate is available.

In the plotting of the profiles of figure 5 it has been assumed that the variances of the supposed normal distributions of P for the various regions are the

same. If this were true then the differential increments in venation potential on the probit scale found for the various regions would represent true differences. However, if the array of chance factors that operate to cause the spread of P around $\overline{P}$ for a genotype were to vary from region to region, these nongenetic factors in any one region might be such as to produce a curve of variation of P with a relatively large standard deviation (terminal intervals) as compared with other regions (central intervals). If such were the case the same genetic difference (e.g., *ci/ci* vs. *ci/–*) would be expected to have a smaller relative effect in the terminal regions than in the central regions. Utilizing a method devised by WRIGHT (1934) it is possible to calculate the standard deviations of the distributions of P that must hold at various intervals of the fourth vein if the increments at each interval are to be made equivalent.

The latter goal can be accomplished by transforming the inverse probability representation in each region from the scale on which the standard deviation is the unit of measurement (scale a) to a new scale, c, on which the difference between the means for *ci/ci* and *ci/–* expressed on the a scale becomes unity. The standard deviations for each region in terms of the c scale can then be calculated by taking the reciprocal of the difference between the *ci/ci* and *ci/–* means on the a scale. Placing the origin (o) of the c scale at the threshold, P_T, the means for *ci/ci* and *ci/–* in each region on this scale are given by the product of the standard deviation on the c scale and the inverse probability function of the venation frequency for the given region and genotype. The means and standard deviations calculated under the above assumptions are given in figure 7 for various regions of the fourth vein. In view of the alternative interpretations of the differential responses of different regions of L_4 to increase in dosage of the *ci* allele it does not seem profitable to speculate further on the nature of primary *ci* action in terms of the present data.

However, regardless of the nature of the primary action at the loci involved, or of the exact additive nature of the interactions revealed by these studies, the present investigation has demonstrated considerable enhancement of mutant actions in combination as compared to their actions considered singly. Such augmentation of effect is common in the genetic literature, and has often been interpreted, as in the present case, in terms of cumulative action of subthreshold effects. To quote PLUNKETT (1926), when speaking of the effects of modifying genes on bristle reduction, " But the effects of these ' weak ' genes are too slight to be noticed in the absence of other ' stronger ' bristle-reducing genes, except by careful counts of all the bristles in a large number of flies. In combination with the main bristle-reducing genes, however, the effects of these minor modifiers are disproportionately enhanced." And later, concerning main gene effects, " This is exactly what happens also when any two of the ' main genes ' are combined. In general, each of these genes disproportionately intensifies the effect of any other one when combined with it; i.e., the reduction of mean bristle numbers effected by the combination is greater than the sum of the reductions effected by the two genes separately. In many cases a gene which alone has apparently no effect on a particular bristle greatly intensifies

the effectiveness of another gene in removing that bristle. In some cases two genes, neither of which, separately, ever removes a certain bristle, do so when combined . . . ." Similarly, DUNN (1942) in speaking of the spotting phenotype in the house mouse, says, " Many mutations, however, produce so small a change in the reactions leading to spotting that the threshold of visible expression is not reached." And further, " Such subthreshold alleles therefore act as modifiers of other factors with more extensive effects by interacting additively with them." NEEL (1941) and WADDINGTON (1939) have also reported similar phenomena. The former investigator found that polychaetoid (*pyd*) produces a considerable enhancement of the wing hair effect of hairy (*h*), although *pyd* by itself had no visible effect on wing hairs. WADDINGTON postulated a subthreshold effect on tarsus segmentation for the ss^a allele, which alone has no visible effect on segmentation, the subthreshold interpretation being based on the extreme enhancement of segmentation found in compounds of ss^a with other aristopedia alleles that produce tarsal segmentation.

While the enhancement of mutant action in combination may be the result of new interaction patterns unique to the combination, the strong possibility exists that the augmentation of effect seen in these combinations represents a transgression of thresholds of morphological effect by reinforcement of subthreshold actions on the biochemical level. If, as CASPARI (1949) has suggested, developmental processes operate under a certain margin of safety, the observed effects of a particular gene substitution may be much more extensive than the spectrum of its morphological effects reveals. The operation of protective thresholds may prevent certain tissues and organs from being affected at the morphological level by a given gene change, although these systems in the mutant may actually differ from the wild type at a subthreshold level. The operation of such thresholds would thus mask a portion of a mutant's pleiotropic effects, introducing distortion into any conception of the total role played by the gene in the control of developmental processes. The undetected presence of such subthreshold components of gene action might also lead to severe underestimates of the evolutionary role played by certain mutants.

Studies which concentrate on the manipulation of genetic variables at one locus only, keeping the rest of the genotype constant, suffer from the disadvantage of maintaining constant some of the factors which control the very thresholds that obscure a part of the gene's action spectrum. It is perhaps one of the outstanding merits of the interaction approach that by producing disturbances in the gene complex at various points, singly and in combination, these threshold relationships may be altered and a larger extent of a mutant's spectrum of effects so brought under observation.

SUMMARY

The interactions of Hairless, engrailed and cubitus interruptus have been investigated at 26°C relative to their interruption effects on the fourth longitudinal vein of *Drosophila melanogaster*.

The data show general agreement with an interpretation of additive action

of the mutants in combination if there is assumed cumulative action of subthreshold components of mutant action.

The pattern of L_4 interruption characteristic of the various genotypes studied has been analyzed by determining the frequency of venation at each of twenty intervals along the length of the fourth vein and by transforming these frequencies to inverse probability functions.

A study of these transformed frequency profiles reveals subthreshold actions of the three mutants in various regions of the fourth vein, as well as differential responses of L_4 venation to changes in dosage at the *ci* locus.

These findings are discussed in relation to the nature of the vein-forming reaction in the Drosophila wing.

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V-TYPE POSITION EFFECTS OF A GENE IN DROSOPHILA VIRILIS NORMALLY LOCATED IN HETEROCHROMATIN[1]

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THE general problem of the relationship between stable chromosome rearrangements and somatic variegation has been reviewed recently by LEWIS (1950) and HANNAH (1951). Although many cases in Drosophila are known in which the variegation is caused by placing heterochromatin adjacent to or near genes normally located in euchromatic regions, the only extensive study that has been made of the factors responsible for variegation of characters determined by genes normally located in heterochromatin is that of KHVOSTOVA (1939) on the cubitus interruptus (*ci*) gene in *Drosophila melanogaster*. In the work being reported, 32 different chromosome rearrangements in *Drosophila virilis* are described, each of which produces variegation of the eye pigmentation when heterozygous with the recessive allele of the gene peach whose locus is normally in heterochromatin. An abstract of a portion of this work has been published (BAKER 1952b).

This gene is the closest known locus to the centromere of chromosome 5, being 203 crossover units from the tip of this apparently telocentric chromosome. Two recessive alleles of the gene are known, pe^{Jap} and *pe*. The latter, when homozygous, produces a lighter eye color than the former and is the allele used in practically all the crosses to be discussed. All the rearrangements responsible for the peach-mottled eyes were induced by X-rays in sperm of wild-type males of the Pasadena strain. The majority of these R(+) rearrangements (pe^{m5} through pe^{m53}) came from treatments of 5000 r.

RESULTS

Prior to describing the rearrangements which evoke the variegation, a few general remarks which apply to all cases of mottling are in order. In every case tested thus far, the mottling is recessive, i.e., flies of the genotype R(+)/+ are wild type, and R(+)/*pe* have variegated eyes. The eye in most of the mottles appears to be a mixture of wild-type and peach pigmentation, although in some cases (frequently in pe^{m1}) the darkly pigmented areas are definitely darker than wild type. The pattern of variegation is not wholly consistent in the two eyes of a single individual or among flies having the same rearrangement, and therefore it is impossible to differentiate the various mottles by their phenotypic expression (see fig. 1). In general, the pattern may be characterized by

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stating that the variegated eyes are composed of a few patches of mutant or wild-type tissue in the complementary background rather than a salt-and-pepper distribution of the pigmentation. There is little evidence of diffusion of pigments from one ommatidium to an adjacent one, each ommatidium being either mutant or wild type. The pattern of variegation usually extends across

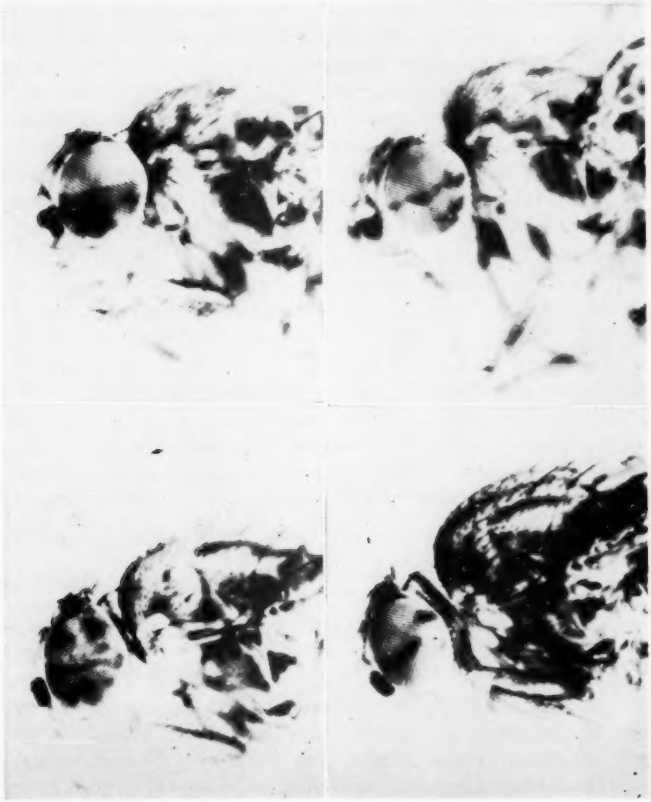


FIGURE 1.—Variations in the phenotypic expression of $T(Y;5)pe^{m1}$. The dark areas in the eye have wild-type pigmentation whereas the light areas are peach.

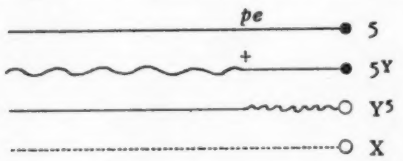
the eye in the anterior-posterior direction; no clearly dorsal-ventral patterns have ever been observed. A line separating the dorsal and ventral halves of the eye appears to influence the pigment distribution since two of the most common patterns are ones in which the dorsal half of the eye is one color and the ventral half the other color (fig. 1) and ones in which all the eye is peach (or wild type) with the exception of a line of facets separating the dorsal and ventral halves (fig. 1). In the majority of mottles the variegation is not so striking as

in the case of pe^{m1}. In many cases most of the eye is wild type with only one or a few small patches of peach pigmentation.

Peach-mottled 1

This first case of variegation involving the *pe* locus was discovered in an F_1 male from the progeny of irradiated wild-type males which were crossed to *b; t; cd; pe* (markers on chromosomes 2, 3, 4 and 5, respectively) females. When this male was testcrossed to the marked females, there was no evidence of linkage between chromosomes 2, 3 or 4, but it could be inferred that a translocation had taken place between the Y and chromosome 5 since all the females resulting from the testcross were phenotypically peach and most of the males were mottled. A similar testcross made at a later time gave the following results in a generation composed of 4103 flies: 51.7 percent peach females, 39.7 percent mottled males, and 8.6 percent peach males. Although these data indicate the presence of a Y; 5 translocation, a careful study of the salivary gland chromosomes and metaphase configurations found in larval ganglion cells failed to reveal any chromosome abnormalities. Therefore, both chromosomes have been broken at approximately the same distance from their centromeres.

In order to elucidate the structure of the translocation, mottled males were crossed to wild-type females and the resulting F_1 males were crossed to peach females. The results of this cross and the subsequent tests made on the F_2 offspring are shown in table 1. If it is assumed that chromosome 5 has been broken distal to the *pe* locus and that the 5^Y chromosome is responsible for the variegation, then the results shown in this table become understandable. Thus the chromosome configuration of a mottled male is assumed to be as follows:



The F_1 wild-type males would have the same configuration, but the wild-type allele of peach would be on the normal chromosome 5. Alternate segregation in the F_1 male translocation heterozygote would produce mottled males and wild-type females when mated to peach females, while adjacent-2 segregation would give wild-type males and peach females if flies which are hyperploid or hypoploid for the proximal region of 5 are viable. The validity of this interpretation is established by the offspring of crosses (2) and (3), which show that the F_2 wild-type males carry the 5^Y chromosome which produces the variegation. They further indicate that the F_2 peach females carry the Y^5 chromosome which has a deletion for the *pe* locus. It should be noted that, genetically or cytologically, it is impossible to differentiate between the Y^5 chromosome and a normal chromosome 5 carrying the *pe* allele. Thus it is impossible to distinguish between mottled males of the type $5/Y^5/5^Y$ and those of the constitution $5/5/5^Y$. After it was found that $5/5/5^Y$ males carried the translocated

chromosome causing variegation and were fully fertile, stocks of this translocation were maintained by mating the mottled male offspring (from the cross peach females to F_2 wild-type males) to peach females. Thus all the mottled stocks are of a known constitution, namely, $5/5/5^Y$.

The F_2 peach males remain unaccounted for on this explanation. It is obvious from cross (4) that they contain the 5^Y chromosome, but phenotypically the mottling has gone to completion (i.e., all the eye is mutant tissue) since in every case they produced mottled male offspring when crossed to peach females.

It is interesting to note that, when the peach males which are genetically like the mottled males are added to the latter class, adjacent-2 segregation and alternate segregation are equally frequent in this translocation heterozygote. In addition, it has been found that 3-1 segregation is almost entirely absent.

TABLE 1

Crosses verifying the constitution of pe^{m1}.*

Cross	Mottled ♂♂ × wild-type ♀♀ F_1 are all wild type					
	Percentage of offspring in the various classes					
	Wild ♂♂	Mot ♂♂	pe ♂♂	Wild ♀♀	pe ♀♀	Total No.
(1) F_1 wild ♂♂ × pe ♀♀	25.1	17.9	6.3	25.6	25.0	9363
(2) F_2 wild ♂♂ × pe ♀♀	26.3	20.3	2.2	26.8	24.3	3561
(3) F_2 pe ♀♀ × pe ♂♂			~50		~50	Not counted
(4) F_2 pe ♂ × pe ♀♀†		36.7	11.4		51.9	3233

*The author is grateful to Mrs. Chloris Dow, who gathered the data given in this table

†Data from 27 individual males.

This type of segregation should produce mottled females with a chromosome configuration identical to that shown above with the exception that they would have two X chromosomes. Many thousands of flies have been examined from crosses of mottled males to peach females and only one mottled female has been found. When crossed to peach males, she produced 281 offspring consisting of 23.5 percent mottled males, 23.8 percent peach males, 22.0 percent mottled females, and 30.6 percent peach females. Cytological preparations of the ganglion cells from some of the F_1 male larvae from this cross revealed the presence of an extra chromosome. This would be expected, since all the F_1 mottled males would have a normal Y chromosome in addition to the Y involved in the translocation. The presence of this extra Y produces no striking effect on the amount of mutant tissue present in the variegated eyes.

Peach-mottled 15

The variegation in this case is also the result of a translocation, which cannot be observed in salivary chromosomes, between chromosome 5 and the Y. When mottled males were crossed to peach females, the following phenotypes

were observed with the indicated frequencies among the 559 progeny: mottled males, 30.6 percent; peach females, 32.4 percent; mottled females, 19.9 percent; and peach males, 17.2 percent. The relatively low frequency of mottled females and peach males is an indication of the presence of a Y; 5 translocation. Since the F_1 peach males were all completely sterile, it would be suspected that they arose from aneuploid gametes which lack all or most of the Y chromosome. Thus it would be anticipated that the F_1 mottled females arose from nondisjunction of the X chromosome and the translocated chromosome carrying 5 attached to most of the Y. The fact that these females are mottled would indicate that chromosome 5 is broken proximal to the *pe* locus. In order to verify the location of the break in chromosome 5, mottled males were crossed individually to wild-type females, and the resulting F_1 males were crossed to peach females. Since no peach flies were observed among the progeny of the latter cross, it is clear that the break is proximal to the *pe* locus. One interesting fact became apparent upon examination of the ganglion chromosomes in the F_1 male larvae from the above cross. Some of the parental males produced male offspring, all of which had only nine rod-shaped chromosomes, eight normal and one long. Other parental males produced both males of this chromosome constitution and ones with one long, one short and eight normal rod-shaped chromosomes. The males bearing only nine rod chromosomes proved to be fertile. It is thus evident that the translocated chromosome, bearing the tip of the Y attached to the centromere of 5, is not necessary for viability or fertility in the male. From a consideration of pe^{m1} and pe^{m15}, it is apparent that neither the tip of the Y nor the region near its centromere is essential for male fertility.

CYTOLOGICAL ANALYSIS OF REARRANGEMENTS

It is not practicable to give a complete description of the phenotypic expression and genetic tests which have been accomplished on each of the rearrangements. It is sufficient to say that all the mottles have been tested genetically to determine whether any translocations have taken place between chromosomes 2, 3, 4, 5 or 6 which were not observed cytologically. Such tests are necessary since, as will be seen from the following descriptions, one break in chromosome 5 invariably is in the basal heterochromatin and, if this is accompanied by a break in another basal heterochromatic region, the rearrangement may not be observable in salivary gland preparations. In every case the genetic tests verified the cytological picture although additional breakage points (in heterochromatin) were indicated in certain rearrangements.

Cytological preparations of the larval ganglion and salivary gland chromosomes were made using the squash technique. The salivary glands were placed in 1 *N* HCl for 30 seconds before staining with aceto-orcein. Permanent slides were made of all rearrangements using a freezing-drying technique made available to the author by Dr. A. D. Conger (Baker 1952a). The regions of the salivary chromosomes denoted in the following descriptions are taken from the chromosome map of this species prepared by Griffen (Patterson *et al.*

1940). In denoting the manner in which the broken ends are joined in the rearrangement, the free end of the chromosome is shown at the left and the centromere end at the right. The chromosomes are separated from each other by semicolons, the rejoined breaks within a chromosome are separated by commas.

Peach-mottled 1 Baker 47i24 $T(Y;5)pe^{m1}$. No rearrangement is visible in either salivary gland or ganglion chromosomes.

Peach-mottled 2 Baker 51b26 $T(3;5)pe^{m2}$. Two breaks in 5: between 5A7a and b, and between 5H1d and 5H2a. One break in 3 between 3C6a and b. Rearrangement: 3C6a-b to 5H1d-5H2a; 5A7a-b to 5H1d-5H2a, 5A7a-b to 3C6a-b.

Peach-mottled 3 Baker 51c2 $T(4;5)pe^{m3}$. No rearrangement is visible in the salivary gland chromosomes.

Peach-mottled 4 Baker 51c30 $T(3;5)pe^{m4}$. One break in 5 between 5H2d and centromere. One break in 3 between 3B2e and h. Rearrangement: 3B2e-h to 5H2d; 5H2d to 3B2e-h.

Peach-mottled 5 Baker 52a25 $T(3;5)pe^{m5}$. Three breaks in 5: between 5G6c and 5G7a, between 5H1b and 5H2a, and presumably between 5H2d and centromere. One break in 3, presumably in 3H1. (The proximal break in 5 cannot be observed cytologically, and the only cytological evidence of the break in 3 is the high frequency of asynapsis in the basal end of this chromosome. However the genetic evidence clearly indicates a translocation between 3 and 5.) Rearrangement: 3H1 to 5H2d; 5G6c-5G7a to 5H1b-5H2a, 5G6c-5G7a to 5H1b-5H2a, 5H2d to 3H1.

Peach-mottled 11 Baker 52a28 $T(2;5)pe^{m11}$. One break in 5 between 5H2d and centromere. One break in 2 between 2A4b and e. Rearrangement: 2A4b-e to 5H2d; 5H2d to 2A4b-e.

Peach-mottled 12 Baker 52a29 $T(2;5)pe^{m12}$. One break in 5 between 5H2d and centromere. One break in 2 between 2B8a and d. Rearrangement: 2B8a-d to 5H2d; 5H2d to 2B8a-d.

Peach-mottled 15 Baker 52a29 $T(Y;5)pe^{m15}$. No rearrangement is visible in salivary gland chromosomes, but one long and one short chromosome are evident in ganglion cells.

Peach-mottled 16 Baker 52a29 $T(4;5)pe^{m16}$. One break in 5 between 5H2d and centromere. One break in 4 between 4D1b and e. Rearrangement: 4D1b-e to 5H2d; 5H2d to 4D1b-e.

Peach-mottled 18 Baker 52a30 $T(4;5)pe^{m18}$. Two breaks in 5: between 5B2f and g, and between 5H2d and centromere. One break in 4 between 4F1c and d. Rearrangement: 4F1c-d to 5H2d; 5B2f-g to 5H2d, 5B2f-g to 4F1c-d.

Peach-mottled 19 Baker 52a30 $T(2;3;5)pe^{m19}$. Two breaks in 5: between 5G4a and b, and between 5H2d and centromere. One break in 3 in proximal end of region 3A1. One break in 2 in 2H2 region. Rearrangement: 5G4a-b to 5H2d; 2H2 to 5G4a-b, 5H2d to 3A1; 3A1 to 2H2.

Peach-mottled 20 Baker 52b2 $T(2;5)pe^{m20}$. Three breaks in 5: between 5C5f and g, between 5H1d and 5H2a, and between 5H2d and centromere. One

break in 2 between 2C1a and b. Rearrangement: 2C1a-b to 5H1d-5H2a, 5C5f-g to 5H2d; 5C5f-g to 5H1d-5H2a, 5H2d to 2C1a-b.

Peach-mottled 22 Baker 52f12 $T(4;5)pe^{m22}$. One break in 5 between 5H2d and centromere. One break in 4 between 4A6a and c. Rearrangement: 4A6a-c to 5H2d; 5H2d to 4A6a-c.

Peach-mottled 24 Baker 52f12 $T(1;5)pe^{m24}$. One break in 5 between 5H2d and centromere. One break in 1 between 1E5b and e. Rearrangement: 1E5b-e to 5H2d; 5H2d to 1E5b-e.

Peach-mottled 25 Baker 52f14 $T(4;5)pe^{m25}$. Two breaks in 5: between 5B4d and f, and between 5H2d and centromere. One break in 4 between 4A3a and c. Rearrangement: 5B4d-f to 5H2d; 4A3a-c to 5H2d, 5B4d-f to 4A3a-c.

Peach-mottled 29 Baker 52f16 $T(3;5)pe^{m29}$. One break in 5 probably between 5H2d and centromere. One break in 3 between 3C5a and c. Rearrangement: 3C5a-c to 5H2d; 5H2d to 3C5a-c.

Peach-mottled 31 Baker 52f16 $T(4;5)pe^{m31}$. One break in 5 between 5H2d and centromere. One break in 4 between 4D1a and e. Rearrangement: 4D1a-e to 5H2d; 5H2d to 4D1a-e.

Peach-mottled 32 Baker 52f16 $T(1;2;5)pe^{m32}$. Two breaks in 5: between 5G5c and d, and between 5H2d and centromere. One break in 1 between 1D2a and d. One break in 2 between 2E8a and c. Rearrangement: 1D2a-d to 5H2d; 2E8a-c to 5G5c-d, 5H2d to 1D2a-d; 5G5c-d to 2E8a-c.

Peach-mottled 33 Baker 52f16 $T(1;4;5)pe^{m33}$. One break in 5 between 5H2d and centromere. Two breaks in 1: between 1F2c and 1F3a, and between 1G3h and 1G4a. Two breaks in 4: between 4D5a and d, and between 4G2b and e. Rearrangement: 4D5a-d to 1G3h-1G4a, 1F2c-1F3a to 5H2d; 5H2d to 4D5a-d, 4G2b-e to 1G3h-1G4a; 1F2c-1F3a to 4G2b-e.

Peach-mottled 34 Baker 52f16 $T(4;5)pe^{m34}$. One break in 5 between 5H2d and centromere. Two breaks in 4: between 4E1g and h, and between 4H3b and d. Rearrangement: 5H2d to 4H3b-d, 4E1g-h to 5H2d; 4E1g-h to 4H3b-d.

Peach-mottled 35 Baker 52f16 $T(2;5)pe^{m35}$. One break in 5 between 5H2d and centromere. One break in 2 between 2D4b and d. Rearrangement: 2D4b-d to 5H2d; 5H2d to 2D4b-d.

Peach-mottled 36 Baker 52f16 $T(2;5)pe^{m36}$. One break in 5 between 5H2d and centromere. One break in 2 betwen 2A2c and e. Rearrangement: 2A2c-e to 5H2d; 5H2d to 2A2c-e.

Peach-mottled 37 Baker 52f16 $T(2;5)pe^{m37}$. Two breaks in 5: between 5B5b and 5C1a, and between 5H2d and centromere. One break in 2 between 2B2g and 2B3e. Rearrangement: 2B2g-2B3e to 5H2d; 5B5b-5C1a to 5H2d, 5B5b-5C1a to 2B2g-2B3e.

Peach-mottled 38 Baker 52f16 $T(1;5)pe^{m38}$. One break in 5 between 5H2d and centromere. One break in 1 between 1D8f and 1D9b. Rearrangement: 1D8f-1D9b to 5H2d; 5H2d to 1D8f-1D9b.

Peach-mottled 39 Baker 52f18 $T(2;5)pe^{m39}$. One break in 5 between 5H2d and centromere. One break in 2 between 2D2g and 2D3a. Rearrangement: 2D2g-2D3a to 5H2d; 5H2d to 2D2g-2D3a.

Peach-mottled 41 Baker 52f18 $T(4;5)pe^{m41}$. Two breaks in 5: between 5E6e and 5E7a, and between 5H2d and centromere. One break in 4 between 4D2i and 4D3c. Rearrangement: 5E6e-5E7a to 5H2d; 4D2i-4D3c to 5E6e-5E7a, 5H2d to 4D2i-4D3c.

Peach-mottled 42 Baker 52f18 $T(4;5)pe^{m42}$. One break in 5 between 5H2d and centromere. One break in 4 between 4B3c and 4B4a. Rearrangement: 4B3c-4B4a to 5H2d; 5H2d to 4B3c-4B4a.

Peach-mottled 45 Baker 52f19 $T(1;5)pe^{m45}$. One break in 5 between 5H2d and centromere. One break in 1 between 1C1a and c. Rearrangement: 1C1a-c to 5H2d; 5H2d to 1C1a-c.

Peach-mottled 46 Baker 52f19 $T(1;5)\ pe^{m46}$. One break in 5 between 5H2d and centromere. One break in 1 between 1C7b and d. Rearrangement: 1C7b-d to 5H2d; 5H2d to 1C7b-d.

Peach-mottled 50 Baker 52f25 $T(1;5)pe^{m50}$. Two breaks in 5: between 5A3f and i, and between 5H2d and centromere. One break in 1 between 1E2c and d. Rearrangement: 5A3f-i to 5H2d; 1E2c-d to 5H2d, 5A3f-i to 1E2c-d.

Peach-mottled 51 Baker 52f25 $T(3;5)pe^{m51}$. One break in 5 between 5H2d and centromere. One break in 3 between 3B2c and d. Rearrangement: 3B2c-d to 5H2d; 5H2d to 3B2c-d.

Peach-mottled 53 Baker 52f26 $T(3;5)pe^{m53}$. One break in 5 between 5H2d and centromere. One break in 3 between 3B2a and b. Rearrangement: 3B2a-b to 5H2d; 5H2d to 3B2a-b.

Evidence that pe is normally in heterochromatin

The evidence that the *pe* locus is contained within the last four bands of heterochromatin at the base of chromosome 5 (see plate I A) comes from an analysis of three rearrangements, pe^{m1}, pe^{m3} and pe^{m4}. The conclusive evidence in the case of pe^{m1} that the break in chromosome 5 is distal to the locus has been stated. However, a searching analysis of the salivary chromosomes has failed to reveal any abnormality. Similarly, pe^{m3} shows no visible rearrangement in the salivary chromosomes although genetic evidence indicates the presence of a translocation between chromosomes 4 and 5 since the offspring of a testcross to *cd; pe* consist of the following: mottled males and females, 1564; cardinal-peach males and females, 1341; and peach males, 1. In order to determine whether the break in chromosome 5 is distal or proximal to the *pe* locus, mottled males were crossed to wild-type females, and the resulting F_1 males were mated individually to peach females. The following types were found among the 2030 progeny produced from 14 F_1 males which carried the translocated chromosome inducing variegation: 52.6 percent wild type, 22.5 percent mottled, and 24.8 percent peach. The peach progeny can come either from nondisjunctional gametes formed in the $5/5^4/4^5/4$ F_1 males or from disjunctional gametes produced by F_1 males having the constitution $5/5/5^4/4$. In either case, the peach phenotype of such individuals indicates that the break is distal to the *pe* locus. Evidence corroborating this finding can be seen in the testcrosses to *cd; pe* mentioned above. If the break were proximal to the locus,

peach and cardinal-peach-mottled flies should have been observed in appreciable frequencies, but such was not the case. The fact that, in both these rearrangements it has been shown that the break is distal to the gene yet no abnormalities in the salivary chromosomes are observed, indicates that the distal limit of the region in which this locus is contained is the first of the four dark bands of basal heterochromatin. If the break were distal to this point, it could be detected easily.

The proximal limit of the region containing the *pe* locus can be deduced from crosses involving pe^{m4}. In crosses of this mottle to peach, a few abnormal flies having peach eyes are always produced. Cytological examinations of the ganglion and salivary chromosomes from progeny of these abnormal flies show that the tip of chromosome 3 is present in triplicate, i.e., the constitution of the abnormal flies is $5^3/3/3$. The fact that these flies, which arise from 3-1 segregation in the translocation heterozygote, are invariably peach in phenotype indicates that the break is proximal to the *pe* locus. Cytologically, the break is between the last band of heterochromatin and the centromere, thus marking the proximal limit of the region within which *pe* is contained.

Relationship between variegation and heterochromatin

Since it has been established that the *pe* locus is in heterochromatin, it is instructive to determine the euchromatic-heterochromatic relationships which can evoke variegation. In the case of rearrangements pe^{m1} and pe^{m3}, it is obvious that foreign heterochromatin from either the Y chromosome or the base of chromosome 4 will cause the mottling when placed distal to the locus. It is also evident from pe^{m15} and pe^{m34} that foreign heterochromatin from the Y chromosome or the 4H3 region, when located proximal to the last four bands of heterochromatin of 5 and thus proximal to the locus, will induce variegation (see plate I B). Euchromatin placed proximal to the locus will also induce variegation as made evident by peach-mottled numbers *4* (see plate I C), *11, 12, 16, 18, 19, 22, 24, 25, 31, 32, 33, 35, 36, 37, 38, 39, 41, 42* (see plate I D), *45, 46, 50, 51* and *53*. The pe^{m2} rearrangement is the only case so far detected in which the presence of foreign euchromatin distal to the locus is responsible for the variegation (see plate I E). It is thus apparent that a disruption of the normal chromosome continuity on either side of the *pe* locus by substitution of either foreign heterochromatin or euchromatin is capable of evoking variegation.

Distribution of breaks associated with variegation

It is clear from an examination of the rearrangements associated with variegation that all involve at least a single break on one side or the other of the 5H2 region. It is also evident that most of the rearrangements involve the relocation of euchromatin to the proximal side of the *pe* locus. In this regard it is interesting to see if the euchromatin responsible for the mottling is randomly distributed among the chromosomes. Figure 2 is a diagrammatic map showing the relative length of the salivary chromosomes and the points of

breakage of the rearrangements. If both the breakage points brought into juxtaposition to the 5H2 region and the other associated breaks in the rearrangements are considered, it can be shown that there is no evidence of nonrandom distribution throughout chromosomes 1, 2, 4 and 5. However, if only the breaks brought into association with the *pe* locus are considered, there is definite evidence of nonrandom distribution in chromosomes 2 and 3. For example, in the latter chromosome, three of the six breaks found are within seven bands of each other. This raises the possibility that either these regions are particularly susceptible to breakage or that they are unique regions which, when placed next to *pe*, affect the expression of this locus. It should also be noted that relatively few breaks associated with variegation are observed close to the

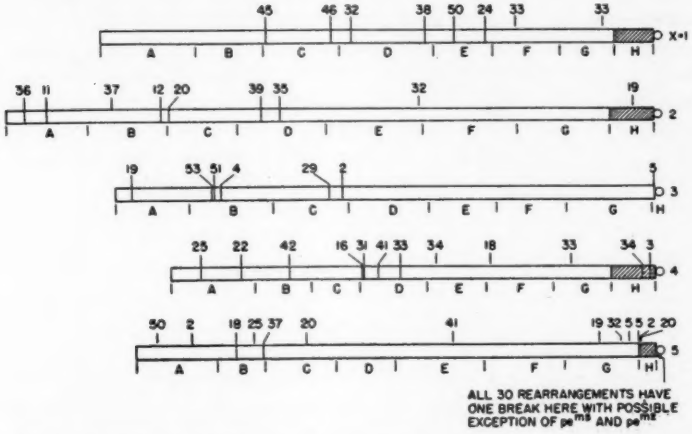


FIGURE 2.—Breakage distribution in salivary gland chromosomes of pe^{m} rearrangements. The shaded areas indicate the basal heterochromatic regions. For a given rearrangement, the line passing through the chromosome indicates the breakage point brought close to *pe*, the other lines indicate accompanying breaks.

proximal ends of the chromosomes. A similar nonrandom distribution of breaks associated with position effects at the *ci* locus in *D. melanogaster* was shown by KHVOSTOVA (1939) to be caused by the fact that other centromeric regions when placed close to *ci* did not influence the action of this gene.

It is evident that the great majority of the rearrangements involve a break between the last salivary band (5H2d) and the centromere. This fact took on added significance when it was discovered that rearrangements causing peach variegation were produced with an extremely high frequency by X-radiation. Pasadena males were given 5000 r and mated to *pe* females, and the F_1 progeny were examined for variegated eyes. Of the 969 F_1 males, 3 peach and 10 mottled males were observed, and of the 869 F_1 females, 22 had variegated eyes. It was possible to establish stocks of 8 of the mottles derived from the F_1 males and 14 of the mottles from the F_1 females. (It should be noted that,

in every case in which the eye pigmentation is variegated, a rearrangement has been detected either cytologically or genetically.) The original mottled flies were crossed to peach flies of the opposite sex, and a count was made of the offspring. It was found, by lumping the data from all rearrangements, that only 36 percent of the F_2 males carrying the translocation (phenotypically wild type or mottled) were actually mottled. Similarly, in the F_2 females 52 percent of the flies which carried the rearranged chromosomes actually showed a detectable variegation in the eye. Therefore, if an estimate of the frequency with which viable rearrangements capable of producing variegation is desired, the penetrance must be taken into consideration. From these figures, it is estimated that about 28 F_1 males and 62 F_1 females carried a rearrangement capable of producing variegation. To these 90 flies should be added the three peach mutants, since the two of these which were fertile showed a deficiency of the 5H2 region. Thus 93 : 1838 or 5 percent carried rearrangements capable of causing variegation, and in the great majority of these the break close to the 5H2 region is between the last band of heterochromatin and the centromere.

This high break frequency within a small region of the salivary chromosome makes it appear that this region is particularly susceptible to breakage by X-rays. However, a more typical picture of the cytological length of this heterochromatic region can be seen in the ganglion chromosomes. Plate 1 F is a photograph of a peach deficiency ($pe^{Df52/23}$) as seen in the salivary gland chromosomes. The deletion is less than one-thirteenth the length of the salivary chromosome. Plate I G shows a prometaphase configuration in the ganglion cells of the same individual. In these chromosomes the deficiency has reduced the chromosome length to about three-fifths its normal size. Therefore, it is apparent that the heterochromatin at the base of chromosome 5, as seen in the salivary glands, comprises from about a third to half the chromosome length in ganglion cells.

DISCUSSION

There seems little doubt that in many cases the variegated type of position effect is in some manner related to simultaneous changes in euchromatic and heterochromatic chromosomal regions. On the basis of work on variegation in *Drosophila melanogaster,* SCHULTZ (1941, MORGAN and SCHULTZ 1942) has set forth a series of general " rules," which appear to hold true for the majority of the then-studied cases of this type of position effect. The rules which are pertinent to this discussion are as follows:

1. Genes normally in euchromatic regions must be placed near heterochromatin in order for variegation to be expressed. The closer the gene is to heterochromatin, the more extensive the variegation.
2. Heterochromatic genes must be placed near euchromatin for the evocation of variegation.
3. In the case of euchromatic genes the addition of extra heterochromatin (either Y chromosomes or heterochromatic regions of the X chromosome or of chromosomes 2 and 4, as well as certain " puff " regions) to

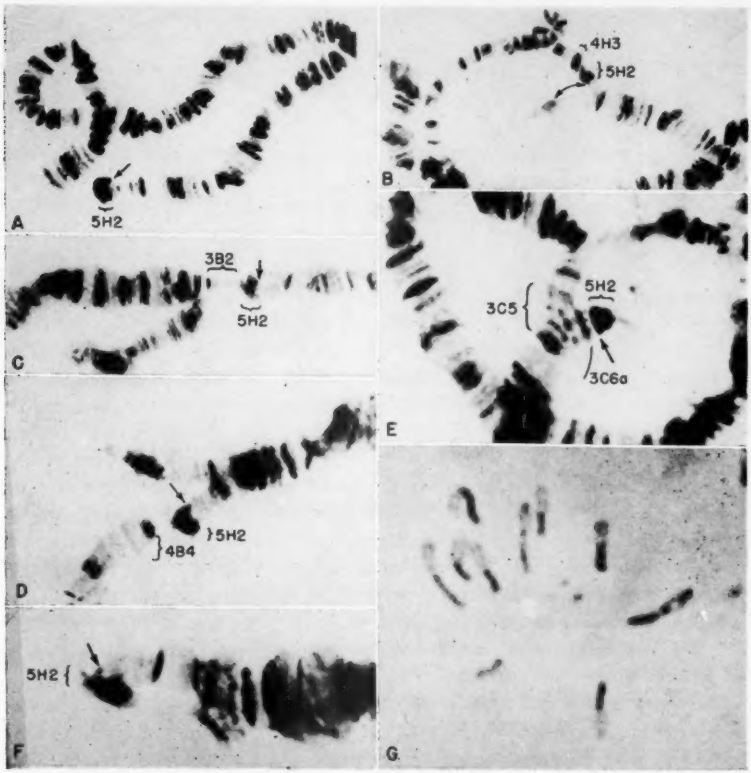


PLATE I

Chromosome rearrangements associated with variegation. In all the photographs the arrow points to the beginning of the 5H2 region within which the *pe* locus is contained.

A.—A normal chromosome 5.

B.—*T(4;5)pe*^{m34}. A portion of the basal heterochromatin of chromosome 4 is placed proximal to the *pe* locus.

C.—*T(3;5)pe*^{m4}. Euchromatin from 3 is located proximal to the *pe* locus.

D.—*T(4;5)pe*^{m42}. Euchromatin from 4 is placed proximal to the *pe* locus.

E.—*T(3;5)pe*^{m3}. Euchromatin from 3 is attached at a point distal to the *pe* locus.

F.—Deficiency *pe*$^{Df\,ras\,fm}$ as seen in salivary gland chromosomes.

G.—Ganglion chromosomes from the same larva as in F. The deficient chromosome is at eight o'clock.

the chromosome complement suppresses the variegation, whereas subtraction of heterochromatin enhances the mottling (see LEWIS 1950 for references).

4. With heterochromatic genes the addition of extra heterochromatin enhances the variegation (or further inactivates the gene), and its subtraction arrests the variegation.
5. When placed near heterochromatin, the euchromatic locus appears to be partially inactivated.
6. The homozygotes of rearrangements, which cause V-type position effects of euchromatic genes, have more extreme variegation than the heterozygotes.

It should be realized that, as far as the heterochromatic loci are concerned, the above rules are based predominantly on studies of a single locus, *lt* (light), located in the centromeric region of chromosome 2L. It is apparent that in *D. virilis* the apposition of a heterochromatic locus (*pe*) to euchromatin is a sufficient but not a necessary condition for the expression of variegation, since foreign heterochromatin will produce mottling equally well. If this observation proves to be generally applicable to heterochromatic loci, then it is evident that variegation is not only dependent upon partial inactivation of a gene brought about by bringing foreign chromatin close to its locus, but it also depends on disrupting the integrity of the heterochromatin. Viewed in this light, there may be no fundamental difference between the factors which evoke variegation in euchromatic and heterochromatic genes. A disruption of the continuity of the heterochromatic regions may be sufficient to produce the mutant phenotype of genes normally located in these regions or in those loci brought close to heterochromatin by rearrangements.

This interpretation of the results may seem at variance with the findings of KHVOSTOVA (1939) at the *ci* locus. She found that a change in action of *ci* is associated with its removal from heterochromatin immediately adjacent to spindle fiber attachment regions. Apparently other basal heterochromatin not immediately adjacent to the centromere, distal regions of the Y chromosome, as well as euchromatic regions are effective in modifying the expression of *ci*. These observations, in conjunction with the finding that other centromeric regions when placed close to *ci* did not produce a position effect, would seem to indicate a difference in the response of heterochromatic and euchromatic loci to a change in position. However, as pointed out by KHVOSTOVA (*loc. cit.*), the regions very close to the centromere may be homologous in all chromosomes. Therefore, rearrangements involving two such regions may not really disturb the continuity of the heterochromatin. It may be that an altered expression of genes located close to the point of breakage is observed only when different heterochromatin or when euchromatin is inserted into the basal heterochromatin.

It will be noted that there are three exceptions among the 32 rearrangements to the general observation that the variegation was accompanied by a break in the heterochromatin between the *pe* locus and the centromere. Al-

though in two cases (pe^{m1} and pe^{m3}) the break in chromosome 5 was distal to *pe*, the break in the other chromosome was in basal heterochromatin. In the third exception, pe^{m2}, one break in chromosome 5 was just distal to the locus while the other breaks were in euchromatin far removed from centromeric regions. The fact that in all the rearrangements showing *ci* position effects studied by KHVOSTOVA the break in chromosome 4 was invariably between *ci* and the centromere, raises the possibility that unobservable inversions in the heterochromatin of chromosome 5 might have accompanied these rearrangements and have been responsible for the appearance of the mutant phenotype in somatic tissue.

Theoretically, the mutant phenotype could be brought about either by a suppression of the gene products of the wild-type allele or by actual mutation or loss of the locus *per se*. Although cases of variegation have been interpreted by some workers to be caused by genetic instability in somatic cells, the stronger evidence points to a somatic disturbance of gene action (BELGOVSKY 1946; GERSH 1952; discussion by LEWIS 1950).

BELGOVSKY (1946) presents the hypothesis that variegation is caused by the interaction of substances produced by affected loci with those produced in heterochromatic regions. It is difficult to see how an interaction of products could explain the results, although a model based on interaction between substrates necessary for normal functioning of heterochromatin and genes is not unreasonable. For example, it might be assumed that the heterochromatin, at least within one region, acts as a unit in the formation of certain cellular constituents, P_H. Certain of the intermediates, I_H, in the formation of products from heterochromatin, require a substrate, X, for completion of a step in the reactions. If it is assumed that X can react with another substrate, Y, which is necessary in the reactions proceeding from the gene to end-product formation, e.g., precursors of the eye pigments, then any increase in the amount of X that can react with Y causes a decrease in the end product of the gene. Formally, this scheme may be diagrammed as follows:

$$\begin{array}{lcl} \text{Heterochromatin} \longrightarrow & I_H + X \longrightarrow & P_H \\ & + & \\ \text{Gene} \longrightarrow & I_G + Y \longrightarrow & P_G \\ & \downarrow & \\ & P_{XY} & \end{array}$$

Thus, any disturbance in the integrity of the heterochromatin would cause less I_H to be formed, allowing X to react with any Y in its immediate vicinity. If the amount of available Y is a limiting factor in the amount of P_G produced, then this amount may approach the threshold value which corresponds to inactivation of the gene concerned.

The author is aware that such a general model is only one of many possible schemes on which the variegation data can be interpreted. Therefore its validity

is unknown and perhaps even doubtful. However, it serves to focus attention on the important problem of interactions between nuclear materials. For example, this scheme predicts that, if extra heterochromatin could be brought into a chromosome complement, then more I_H, and thus more P_G, might be produced which could result in a suppression of variegation. This prediction is apparently fulfilled except for the generalization by SCHULTZ that addition of heterochromatin enhances the variegation involving heterochromatic loci. So far as the author is aware, this generalization is based solely on the effect of different numbers of Y chromosomes on the expression of variegation at the light locus (MORGAN *et al.* 1934; MORGAN and SCHULTZ 1942) and possibly at cubitus interruptus (KHVOSTOVA 1939). MORGAN and SCHULTZ (1942) found that the addition or subtraction of a Y chromosome was without effect in the R(+) rearrangements involving rolled, a locus in the heterochromatin of 2R. The addition of an extra Y to the chromosome complement of the *pe*^{m1} rearrangement (a Y;5 translocation and thus not strictly comparable to the rearrangements at light) has no striking effect on the extent of mottling, although there may be a slight suppression. It should be noted that, according to the model, the addition of extra heterochromatin would be effective in suppressing variegation only if the additional I_H produced could increase the supply of Y by depleting the amount of X present in the *immediate vicinity* of the gene. In certain cases the presence of an extra Y apparently does not affect the variegation of euchromatic genes, e.g., yellow-mottled in *D. virilis* (GIRVIN 1949). SCHULTZ's observation that R(+)/R(+) genotypes show more extensive variegation than the rearrangement heterozygous with the mutant is difficult to explain on this scheme, unless it is assumed that heterochromatin produces some product which is essential in the reactions leading from gene to the production of its end product. None of the mottles which have been obtained in the homozygous condition (*3, 4, 31* and *39*) consistently show more extreme variegation in homozygotes than in heterozygotes.

There is one other line of evidence which lends some credence to the assumption that destruction of the continuity of the heterochromatin is just as necessary a factor in producing variegation as the juxtaposition of a gene to foreign chromatin. It was noted in the preceding section of this paper that most of the induced breaks associated with peach variegation occurred between the last band of salivary heterochromatin and the centromere of chromosome 5. It was also shown that the breakage frequency in this region is very high, owing presumably to the fact that this salivary region is greatly expanded in normal mitotic chromosomes. Unless the unlikely assumption is made that all these breaks are occurring at a localized spot close to the peach gene, it means that breaks anywhere within this relatively long region (which cannot be discerned in the salivary chromosome 5) are effective in evoking variegation. Studies are being made of the crossover frequency between *pe* and the point of breakage in different mottles as well as the distribution of heterochromatin in various rearrangements as seen in the prometaphase stage of ganglion cells. This work should provide direct evidence on the point under discussion.

If it is true that heterochromatic loci may be affected by breakage events taking place at some distance, then the situation is analogous to the spreading effect frequently observed with euchromatic genes. In terms of the gene-action interpretation of variegation, it means that there may be an interaction lengthwise along the chromosome between substrates utilized by the gene and those utilized by heterochromatin. However, this interaction is not effective between homologues because, as DUBININ and SIDOROV (1935) discovered, using the hairy (*h*) locus, R(+)/*h* flies showed partial expression of the mutant condition, but R(*h*)/+ (derived by crossing over from the former) flies were wild type. From crossovers derived from the latter genotype, the wild-type allele can be restored to the rearranged chromosome, and the mutant expression again becomes evident. This orientation along the length of the chromosome limits the interaction to factors localized on the same homologue. The orientation is thus similar to that proposed by LEWIS (1951) for explaining the stable-type position effect, thus suggesting at least one similarity between the two types of position effect.

SUMMARY

Thirty-two R(+) rearrangements induced by X-rays in *Drosophila virilis* are described which produce variegated pigment in the eyes when heterozygous with the recessive allele of the *pe* locus. This gene is the closest known gene to the centromere of chromosome 5, and evidence is presented that its locus resides within the last four bands of basal heterochromatin as seen in the salivary gland chromosome.

Among these rearrangements, examples are found which indicate that any one of the following types of association between euchromatin and heterochromatin is sufficient to induce variegation: (1) placing foreign heterochromatin distal to the *pe* locus, or (2) proximal to the locus; (3) placing euchromatin either distal or (4) proximal to the locus.

The great majority of the rearrangements involve-a break in chromosome 5 between the last salivary band of heterochromatin and the centromere. The high break frequency observed in this region is probably due to the demonstrated fact that the small salivary heterochromatic region comprises at least two-fifths of the length of the normal mitotic chromosome.

It is held that these findings provide evidence that there may be no great difference in the reactions of heterochromatic and euchromatic loci to V-type position effects. Thus, for variegation to be evoked, it is necessary not only that the locus be brought near heterochromatin but also that the continuity of the heterochromatin concerned be interrupted. An interpretation of variegation is given in terms of interaction between necessary substrates of gene and heterochromatin.

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FREQUENCY OF NOTCH MUTATIONS INDUCED IN NORMAL, DUPLICATED AND INVERTED X-CHROMOSOMES OF DROSOPHILA MELANOGASTER

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AMONG spontaneous and X-ray-induced effects at specific loci in *Drosophila melanogaster,* Notch mutations are particularly frequent. The first Notch (*N,* locus 1–3.0) was found by DEXTER (1914), and Notch mutants were early studied and described by MORGAN (1919) and MOHR (1923 and 1932). In more recent years GOTTSCHEWSKI (1937), DEMEREC (1939, 1940 and 1941), and DEMEREC and FANO (1941) have analyzed many different Notch mutants, including a cytological examination of the salivary chromosomes (see descriptions in BRIDGES and BREHME 1944). From these studies much is known about the Notch mutation. Notch is sex-linked, lethal in the male and homozygous female. In the heterozygous condition females characteristically exhibit excisions or notching of the posterior margin of the wings, as well as other less obvious differences from the wild-type condition. All Notches behave genetically as though they were deficiencies. Not only are they male-lethal but they also invariably " uncover " two recessive genes, split (*spl,* 3.0) and facet (*fa,* 3.0), and frequently other nearby genes as well, such as prune (*pn,* 0.8), white (*w,* 1.5), roughest (*rst,* 1.7), diminutive (*dm,* 4.6), and echinus (*ec,* 5.5).

Split and facet alleles affect the size and surface structure of the eye; in addition *spl* typically produces doubled bristles (hence its name), whereas *fa* shows some degree of wing nicking, particularly the allele fa^n. Both *spl* and *fa* are male-viable, but the combination *spl/fa* is wild-type in appearance. Thus, the two genes do not act like alleles. Still, no crossing over occurs between them, and the exact relationships between *N, spl* and *fa* remain unclear.

DEMEREC, SUTTON and HOOVER have analyzed the salivary chromosomes of a large number of Notch mutants, many of which are described in BRIDGES and BREHME (1944). Their analyses showed that many induced as well as spontaneous Notch mutants are associated with a cytological detectable deficiency. With few exceptions the deficiency includes salivary chromosome band 3C7 of BRIDGES' (1938) map. However, in at least three cases, $N^{264\text{-}73}$, $N^{264\text{-}86}$ and $N^{264\text{-}90}$, band 3C7 appears not to be included in the deficiency.

Although many of the same Notches listed in BRIDGES and BREHME have been reported by DEMEREC (1941) and DEMEREC and FANO (1941), a summary of the entire group will be revealing. In table 1 are presented the Notch mutants from BRIDGES and BREHME about which cytological or crossing over data are given. The tabulation shows the percentages of different categories of Notch mutants, both spontaneous and X-ray induced. It may be seen that

spontaneous Notches differ from X-ray-induced ones in two important respects: a) when deficiencies are detectable, those arising spontaneously tend to be shorter than those resulting from irradiation, and b) spontaneous Notches arising in association with translocations or inversions are very rare. Among the Notches not associated with gross rearrangement, a considerable number show no cytologically detectable deficiency. Even when a deficiency is evident, frequently only one band, 3C7, is missing. Indeed, among the X-ray Notches about 30% are deficient for one band or less, even when those non-deficient mutants associated with rearrangements are not considered.

About one-third of all induced Notches are associated with gross rearrangements; however, a few of these (3 out of 36) show a detectable deficiency. Most of the rearrangements, to be sure, bring band 3C7 near heterochromatin, and the resulting Notch mutants may be ascribed to position effect rather than to gene loss. SCHULTZ (cited in BRIDGES and BREHME 1944) demonstrated that the addition of an extra Y chromosome resulted in the production of

TABLE 1

Summary of Notch mutants recorded in BRIDGES and BREHME (1944) about which cytological or crossing over data are given.

Total No.	White-Notch	Notch-dimin.	Cytol. normal	1 band deficient	2–19 bands deficient	20–50 bands deficient	Rearrangements (non-deficient)
				Spontaneous			
29	13.8%	20.7%	23.5%*	23.5%*	53.0%*	0	0
				X-ray-induced			
95	15.8%	22.1%	16.9%	13.7%	17.9%	16.9%	34.7%**

*Only 17 of the 29 spontaneous Notches were studied cytologically.

**Five additional mutants associated with rearrangements (inversions or translocations) have been excluded because some viable non-Notch males were produced.

viable, though sterile, males in the case of three different Notches, each associated with a translocation which brought band 3C7 close to heterochromatin ($N^{264\text{-}6}$, $N^{264\text{-}9}$ and $N^{264\text{-}10}$).

In none of the deficient Notches does the loss exceed 50 bands (DEMEREC 1941). Therefore, deficiencies of greater extent probably act as dominant lethals, and do not survive even in heterozygous condition. POULSON (1945) studied the embryological development of various Notch males, some with no detectable deficiency and some with maximum loss, and he concluded that the lethal action was similar in both types of Notches. However, no correlation exists between the extent of the deficiency and the degree of expression of the Notch phenotype.

The question exists: is a "no-band" Notch the result of a true lethal gene mutation, or, like a detectably deficient Notch, is a cytologically normal Notch associated with a real loss of genetic material, the loss being too slight to be observable in the salivary chromosomes? Since all Notches behave genetically like deficiencies, the latter interpretation is favored. On the other hand DEMEREC

(1941) put forth the view that in cytologically normal Notches the Notch "gene" has been inactivated (lost its specificity) but that no chromatic material is actually missing. The fact that position-effect Notches exist suggests that genes can be inactivated without loss. Probably the best experimental way to distinguish between these two interpretations would be to attempt to produce reverse mutation of various cytologically normal Notch mutants which are not associated with gross rearrangements. A reversible Notch mutant could not easily be regarded as caused by a genetic loss (cf. MULLER 1930). However, even if the mutational nature of some Notch mutants were demonstrated, any lethal mutation, inactivation, or loss of the N^+ locus should give rise to the typical Notch phenotype.

How then are *fa* and *spl* related to *N*? If *spl* and *fa* are both allelic to *N*, then *spl* and *fa* must be regarded as non-lethal mutational changes of the N^+ locus, and cannot be losses. However, at least one lethal *fa* allele has been reported by MULLER and ALTENBERG (1921). Actually, *spl* and *fa* appear not to be alleles, but they possibly could be pseudo-alleles. If so, then the wild-type condition would be spl^+ fa^+ (cf. BAUER 1943). Now, both viable and lethal *spl* and *fa* alleles may be visualized, but lethal *N* mutants would result only from the loss, inactivation, or simultaneous mutation of *both* loci. In this view a mutational Notch would be very rare indeed, except under some hypothesis of chain mutation, which itself appears most unlikely (GOTTSCHEWSKI 1937). In summary, it is difficult to believe that even cytologically normal Notch lethals can result from true gene mutation, and the same may hold for any lethal at a locus where viable alleles are known. On the other hand the occurrence of viable mutants which result from actual loss rather than from mutation has been demonstrated by MULLER (1935) and others. Thus, the entire series of X-ray induced *N* changes could be visualized on the basis of genetic loss of varying extent.

Two duplications are known which possess in common the ability to suppress the Notch phenotype when compounded with Notch mutants. These are Abruptex (*Ax*) and Confluens (*Co*), described in BRIDGES and BREHME (1944). *Ax* is a one-band duplication, having two bands in place of the normally single 3C7 band (MORGAN, SCHULTZ and CURRY 1941). *Ax* itself is expressed mainly by a shortening of one or more longitudinal wing veins. In combination with *N* the shortened wing veins are commonly observed, but the wings are usually not notched. A small percentage of *Ax/N* individuals do possess Notch wings, however. *Co* is a longer duplication than *Ax*, involving bands 3C5 through 3D6 (SCHULTZ 1941). *Co* is expressed by thickened, but not shortened, wing veins; but *Co/N* is nearly wild-type in appearance. It may be noted that *N* itself shows slightly thickened wing veins. MORGAN, SCHULTZ and CURRY (1941) found that crossing over can occur between a deficiency Notch and *Co*, producing a duplication that no longer has two N^+ loci but is otherwise identical with the original *Co* duplication. The *Co* phenotype is then no longer expressed. Therefore, they concluded that the duplication of the Notch locus produces the *Co* phenotype. Since the *Ax* phenotype is different

from *Co*, the Abruptex effect must result from some sort of position effect on band 3C7 rather than from a true duplication of the Notch locus. The extra band in *Ax* may not be 3C7; if it is, then the Notch locus may not be in 3C7.

Despite the large number of Notch mutants produced by DEMEREC, remarkably few quantitative data on the X-ray induction of Notches appear in the literature. Some indication of the Notch mutation frequency following various doses may be derived from the studies of DEMEREC (1934), MULLER (1940), and LEWIS (1945). However, SITKO (1938) demonstrated that the induced Notch mutation rate can be appreciably increased if band 3C7 is brought close to heterochromatin, as occurs in the white-mottled-4 (w^{m4}) inversion.

The present study is an attempt to analyze the X-ray production of Notch mutations by comparing their incidence in structurally normal chromosomes with that in abnormal chromosomes. A given dose of X-rays (5000 r) was applied to Canton-S wild-type males, and the number of induced Notch mutants was determined. A similar dose was then applied to Abruptex males in order to determine what effect the presence of the extra band in *Ax* has on the production of Notch mutants. Finally, the same dose was applied to w^{m4} and roughest-3 (rst^3, an inversion rather similar to w^{m4}) males in order to observe what effect bringing band 3C7 close to heterochromatin by means of inversions has on the production of Notch mutations. In each case any simultaneous occurrence of a white effect accompanying the Notch mutation was recorded. Moreover, a considerable amount of data was obtained regarding the induction of male-viable split mutations.

The results of these experiments show that the Notch mutation rate in wild-type and *Ax* males is similar. Likewise, the incidence of Notches is the same in w^{m4} and rst^3; but in the latter two stocks the frequency of Notch mutants is more than three times higher than that observed in the non-inversion stocks. Thus, *Ax* appears not to contain a duplication of the Notch locus, and nearness of N^+ to heterochromatin markedly increases the frequency of induced Notch mutations. However, no male-viable *spl* mutations were observed.

MATERIALS AND METHODS

Four stocks of *Drosophila melanogaster* were used to provide males for irradiation: 1. Canton-S wild-type, 2. yellow-2, apricot, Abruptex ($y^2\ w^a\ Ax$), 3. white-mottled-4 (w^{m4}), and 4. yellow, roughest-3, bobbed, carnation ($y\ rst^3\ bb\ car$). The latter two stocks contain inversions bringing the Notch locus close to the proximal heterochromatin (see fig. 1). Males from each stock were placed in size 000 ventilated gelatin capsules for irradiation. A dose of 5000 r was given in all cases. Two different X-ray machines were used in the course of the work. The first, used for most of the Canton-S + and for about half of the w^{m4} exposures, was an oil-cooled G.E. Maximar deep therapy machine, operated at 200 KV and 25 ma at 20 cm target distance, unfiltered. This setting produced an X-ray intensity of 650 r/min as measured by a Victoreen dosimeter, corrected for the barometric pressure. This correction, usually negligible, is significant at the altitude of Salt Lake City, Utah: 4366 feet. The

second machine, used for all other exposures, was a Westinghouse Quadracondex water-cooled deep therapy machine operated at 250 KV and 15 ma at 35 cm target distance, unfiltered. This setting produced an intensity of 425 r/min. The mutational similarity of 5000 r doses delivered from each machine was checked by extensive lethal tests. Canton-S + males were exposed to 5000 r from the first machine and mated with Muller-5 females. In 3,970 F_2 cultures checked, 519 recessive sex-linked lethals were found, an incidence of 13.1%. A presumably equivalent dose from the second machine given to *rst*^{3}

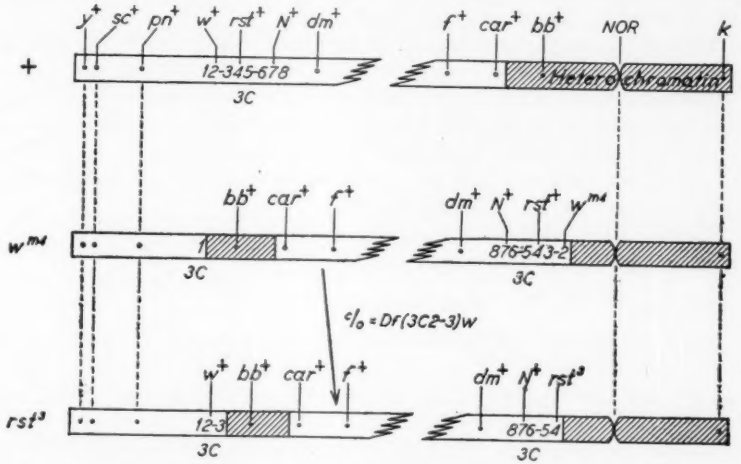

FIGURE 1.—Diagrammatic representation of wild-type, white-mottled-4 (w^{m4}), and roughest-3 (*rst*^{3}) mitotic X-chromosomes, showing the relationships to heterochromatin of various gene loci: yellow (*y*), scute (*sc*), prune (*pn*), white (*w*), roughest (*rst*), Notch (*N*), diminutive (*dm*), forked (*f*), carnation (*car*), and bobbed (*bb*) and also of the nucleolus organizing region (NOR) and the kinetochore (k). The salivary band locations of *w*, *rst*, and *N* are indicated. The euchromatic and heterochromatic break points of the inverted X-chromosomes follow BRIDGES and BREHME (1944) and KAUFMANN (1942 and 1944).

males produced 125 sex-linked lethals in 991 F_2 cultures checked, an incidence of 12.6%. Thus, no significant difference existed in the output of the two machines. Approximately 13.5% sex-linked lethals would be expected from 5000 r, according to MULLER (1950). Possibly the dose actually delivered by the two X-ray machines was somewhat less than 5000 r.

The irradiated males were mated to females whose genotype permitted the detection of white as well as Notch mutations in the F_1 females. In addition, females mated with Canton-S + and *Ax* males possessed inversions in order to prevent crossing over between the irradiated paternal and the maternal X-chromosomes. Since the w^{m4} and *rst*^{3} males themselves had inversions, they were mated with females having normal X-chromosomes, but which carried the recessive gene *spl* in order to facilitate the detection of Notch mutants.

The female F_1 offspring were examined individually for Notch and white mutations under a binocular dissecting microscope at a magnification of 15 or 18 diameters. When a female showing mutant eye color or notching of the wings was found, she was placed in a vial with brothers and allowed to breed. Verification of the mutation was possible when a stock could be developed from the original female for breeding tests. In particular, most of the Notch mutants were proven by mating with special N^+ duplication males. These males possessed a short segment of X-chromosome containing the w^+ and N^+ loci inserted in an autosome. True Notch mutations were covered by this duplication; mimicking mutations were not covered.

However, a number of presumptive Notch and white mutants, having good phenotype, for one reason or another failed to produce offspring for testing. These non-breeding mutants were taken into account in calculating the mutation rates recorded in the next section. A proportion of the sterile mutants equal to the proportion of the fertile mutants which were proven by breeding test to be true white or Notch mutations was added to the proven mutants in order to obtain the overall mutation rates.

Further determined in all cases was the fraction of all Notch mutations which simultaneously affected the white locus.

RESULTS

In table 2 are recorded the complete summarized results of the production of Notch, white and split mutations following irradiation with 5000 r of Canton-S +, *Ax*, w^{m4} and rst^3 males. It may be noted that between 22,000 and 27,000 F_1 females were examined in each series. In table 3 the same data are presented, expressing the frequency of Notch, white and split mutations expected if exactly 25,000 F_1 females had been examined and taking into account the sterile mutants of each kind.

The results as seen in table 3 are clear. The total number of Notch mutations produced in Canton-S + was quite similar to that in *Ax*, being similar moreover to the total number of white mutations in each case. Furthermore, the Notch mutation frequency was the same in w^{m4} as in rst^3, but considerably exceeded that found in either Canton-S + or in *Ax*. It may be further noted that in w^{m4} white-Notches constituted a large fraction of all Notch effects, whereas in rst^3 no white-Notch mutants whatsoever were found because w^+ and N^+ are distantly separated in rst^3, unlike the relationships in w^{m4} (see fig. 1). In w^{m4} those Notches which were not simultaneously white occurred with a frequency slightly, but not significantly, higher than in Canton-S +. However, it must be remembered that detection of *N* mutants was facilitated in w^{m4} (and in rst^3) by the presence of *spl* in the parental females.

It seems fair to combine the Canton-S + and *Ax* data with regard to Notch mutation frequency; likewise the w^{m4} and rst^3 data. Following exposure to 5000 r, then, Notch mutations were produced in the non-inverted chromosomes with a frequency of nearly one per 1000 (44 in 46,570) irradiated X-chromosomes; similarly, white mutations occurred with a frequency of about one per

TABLE 2

Male-viable, male-lethal, and sterile mutants detected in F_1 females following irradiation of Canton-S+, Ax, w^{m4}, and rst^3 males with 5000r.

Mutant	Breeding results	Males irradiated			
		+	*Ax*	w^{m4}	rst^3
White	Viable	6	12	23	10
	Lethal	5	3	14	23
	Sterile	9	7	8	10
White-Notch	Viable	0	0	0	0
	Lethal	3	2	44	0
	Sterile	1	0	16	0
Non-white Notch	Viable	0	0	0	0
	Lethal	12	13	21	58
	Sterile	8	5	4	31
Split	Viable	Not detectable		0	0
No. F_1 ♀♀ examined		24,261	22,309	26,164	26,723

1000 (48 in 46,570). However, in the inversion stocks, Notch mutations occurred with a frequency of one per 300 (174 in 52,887) irradiated X-chromosomes following exposure to 5000 r.

In the F_1 females examined following exposure of w^{m4} and rst^3 males, split as well as Notch mutants could be detected because of the presence of *spl* in the parental females. Altogether, 52,887 F_1 females heterozygous for *spl* were examined but not one male-viable *spl* mutant was observed. Every apparent split effect was, on breeding test, a male-lethal Notch mutant. Of the 174 F_1 females which did have split eyes, 123 were fertile and were proved to be Notch mutants. Among the 51 sterile mutants, it is conceivable that one (or more) might have proved to be a male-viable split mutant, rather than a Notch, had it been fertile. However, the great majority of these sterile mutants showed the typical wing nicking of Notch. Since the fertile ones, whether or not they had obviously notched wings when first observed, invariably bred as

TABLE 3

Expected frequency of all white (male-viable and lethal), white-Notch, non-white Notch, all Notch, and male-viable split mutants following irradiation of Canton-S +, Ax, w^{m4}, and rst^3 males with 5000r, assuming the examination of 25,000 F_1 females and taking into account sterile mutants of each kind.

	Males irradiated	All white mutants	White-Notch mutants	Non-white Notch mutants	All Notch mutants	Viable split mutants
Non-inverted X-chromosomes	+	24.7	4.1	20.6	24.7	....
	Ax	27.0	2.2	20.2	22.4	....
Average		25.8	3.2	20.4	23.6	....
Inverted X-chromosomes	w^{m4}	100.2	57.3	23.9	81.2	0
	rst^3	40.2	0.0	83.3	83.3	0
Average		....	....	....	82.2	0

male-lethal Notch mutants, it seems unlikely that X-rays are effective in inducing male-viable *spl* mutations.

DISCUSSION

Four experimental determinations were made in the present investigation of the X-ray induction of Notch mutations: 1) the frequency of Notch mutations produced in wild-type X-chromosomes by an irradiation of 5000 r, 2) the Notch frequency produced in Abruptex X-chromosomes by the same dose, 3) the Notch frequency in inverted X-chromosomes in which salivary chromosome band 3C7, which presumably contains the N^+ locus, lies close to proximal heterochromatin, and 4) the frequency of male-viable split mutations.

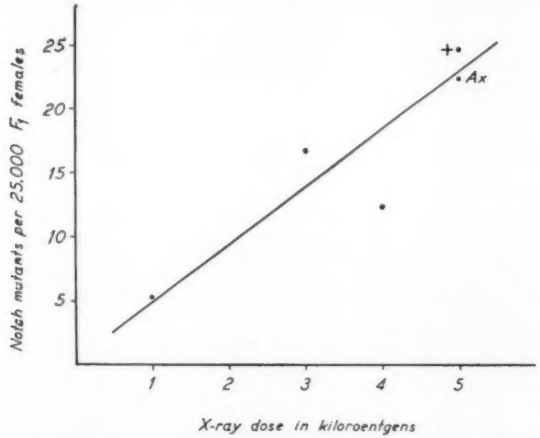

FIGURE 2.—Relationship between X-ray dosage and the production of Notch mutations. The frequency of Notches is expressed as the number expected per 25,000 F_1 females examined. Data at 1000r and 4000r from MULLER (1940), at 3000r from LEWIS (1945), and at 5000r from the present study.

In the irradiation of Canton-S +, Notch mutants occurred with a frequency of approximately one per 1000 irradiated X-chromosomes; one-sixth of the Notch mutations were accompanied by an effect at the white locus. Before these results may be accepted as a basis for comparison with the other irradiations, it is desirable to determine if independent investigations on the frequency of X-ray-induced Notches are in agreement with these results. MULLER (1940) and LEWIS (1945) reported values for the induction of Notches following exposure to 1000 r, 3000 r, and 4000 r. It is not clear whether they included in their values sterile as well as fertile mutants; in any event the detection of Notches was not the primary focus of attention in their experiments. Thus, precise agreement as to the frequency of Notch mutations cannot be expected. However, as can be seen in figure 2, their data combined with those from the present experiment indicate fairly well that Notch mutants are produced with

a frequency proportional to dose. MULLER (1940) believed that mutants such as Notch, even though many are deficiencies, result from the occurrence of single X-ray hits, accompanied by a spread of effect of greater or lesser extent. The combined results depicted in figure 2 support that view for Notch mutations. DEMEREC (1934) also reported data on Notch mutation frequency following exposure to 2500 r. However, not only his values for *N* mutants, but also those for mutants at various other loci, were consistently low in comparison with other studies of mutation frequency. Therefore, his data have not been included in figure 2.

The fraction of Notch mutants which are simultaneously white was not reported by MULLER or LEWIS. However, the Notches listed in BRIDGES and BREHME (1944) may serve for comparison with the present study (see table 1). It must be recognized that two types of accompanying white effects may be present; 1) white eyes, and 2) white-mottled eyes. Only the former, Notches which have pure white eyes, are concerned in this comparison. In the Canton-S + exposures, four of the 24 Notches had white eyes, or 16.7%. Of the 95 cytologically studied induced Notches listed in BRIDGES and BREHME, 15 had white eyes, or 15.8%.

It may be accepted that the results of the Canton-S + irradiation are not significantly different from those of other investigators and may be safely used for comparison with the irradiations of the stocks possessing structurally abnormal X-chromosomes.

The frequency of Notch mutants resulting from the irradiation of *Ax* with 5000 r was quite similar to that produced from Canton-S +, as seen in table 3. However, only two of the 20 Notch mutants found in the *Ax* experiment were white-Notches, while four of the 24 from the Canton-S + irradiation were white-Notch. The numbers are too small to ascribe a significance to this difference. It should be noted that in the combined results of the Canton-S + and *Ax* irradiations six of the 44 induced Notch mutations, or 13.6%, were white-Notch, which is in good agreement with the data from BRIDGES and BREHME (1944).

The fact that the frequency of Notch mutations induced in *Ax* is no different from that in wild-type implies that *Ax* contains no true duplication of the N^+ locus. Thus, the inference of MORGAN, SCHULTZ and CURRY (1941) that *Ax* results from a position effect rather than from a duplication of the N^+ locus is supported by these mutation studies. Nonetheless, *Ax* is effective in suppressing the Notch phenotype in the combination: *Ax*/*N*.

If the extra band in *Ax* suppressed the Notch phenotype as well when *N* is induced in the *Ax* chromosome as it does when *N* is present in the opposite chromosome, then fewer Notches would be expected following irradiation of *Ax* than of wild-type because all no-band and one-band Notches would not be detectable in *Ax*. However, position-effect Notches (showing no deficiency) might well be equally frequent in *Ax* as in + because the position effect could inactivate two adjacent 3C7 bands as well as one. From a consideration of the 95 induced Notches listed in table 1 it may be concluded that the following

three classes of Notch mutants are about equally frequent: a) Notches which are not associated with gross rearrangement and which have a detectable loss of one band or less (29), b) Notches having a detectable loss of two bands or more (33), and c) non-deficient Notches associated with gross rearrangements, giving rise to a position effect (33). Thus, about 30% fewer Notches would be expected from the irradiation of *Ax* than from +.

However, the extra band in *Ax* does not produce a reduction in the detected number of induced Notches; thus, no-band and one-band Notches induced in the *Ax* X-chromosome are not suppressed. Therefore, the usual suppression of the Notch phenotype by *Ax* must result from a position effect on the N^+ locus such that even in a single dose it becomes effective in giving rise to wild-type wings in a female just as a single N^+ locus normally does in a male. The Notch phenotype ordinarily observed in N/N^+ females cannot be a consequence of the absence of one N^+ locus, but rather is the usual expression of a single N^+ locus in a female. The single N^+ locus in the *Ax* chromosome becomes as effective as two N^+ loci in producing normal wings in Ax/N females. However, loss of the N^+ locus from *Ax* must give rise to a Notch phenotype just as it would in a wild-type chromosome because the extra band in *Ax* does not substitute for N^+; that is, the extra band does not contain a duplicated N^+ locus.

Although the *Ax* duplication surely does not contain two N^+ loci, the nature of the extra band present is uncertain. The appearance of the *Ax* salivary chromosome suggests that two 3C7 bands are present (MORGAN, SCHULTZ and CURRY 1941). If so, then the N^+ locus is not located in 3C7. However, the extra band may be some entirely unrelated band which has been inserted next to 3C7. If so, then the N^+ locus may well be contained in band 3C7. No information from the present study serves to distinguish between these two possibilities.

The phenotype associated with a true duplication of N^+ is a thickening of wing veins as seen in Confluens. Moreover, duplications of the N^+ locus which by insertional translocation are present in autosomes, rather than being adjacent to the normal N^+ locus as in Confluens, show a thickened wing vein phenotype similar to that of *Co*. Three such insertional N^+ duplications were used in proving presumptive Notch mutants produced in the present study. All three show a Confluens-like phenotype when present in addition to a normal X-chromosome. However, when present with a Notch mutation in a male, not only may the male survive, but it ordinarily shows neither wing nicking nor Confluens-like wing veins.

In non-inverted X-chromosomes, effects at the Notch locus occurred following 5000 r irradiations with a frequency of approximately one per 1000. However, bringing the N^+ locus close to proximal heterochromatin by means of the w^{m4} and rst^3 inversions increased the frequency of induced Notch mutations more than three-fold. Assuming that the N^+ locus is in band 3C7, then in w^{m4} the N^+ locus is separated from heterochromatin by five bands; in rst^3 by only three bands (see fig. 1). In each of these inversions a position effect is evident

on the expression of genes immediately next to the heterochromatin; however, no change in N^+ is apparent in either inversion. Still, it could be imagined that a position effect on mutability of the N^+ locus is present, even though no effect on phenotypic expression is evident. On the other hand, the proximity of N^+ to heterochromatin may serve to facilitate the production of heterozygous viable deficiencies of the N^+ locus. In normal X-chromosomes, Notch deficiencies exceeding 50 bands do not survive even as heterozygotes (DEMEREC 1941). Should the bulk of deleted material be heterochromatin, however, then deficiencies of much greater actual extent could survive to be detected.

The results of the w^{m4} irradiation serve to distinguish between these two possible interpretations. In w^{m4} two classes of Notch mutations can be observed: a) Notches not simultaneously accompanied by a white effect, and b) white-Notches. If a position effect on mutability of N^+ were present in w^{m4}, then both classes of Notch mutations should be increased in comparison with those produced by irradiating wild-type X-chromosomes. However, the actual data show that nearly all of the increased number of Notch mutations in w^{m4} were white-Notches. Notches which were not white, thus not including any heterochromatic deficiency, occurred with a frequency of about one per 1000, similar to the frequency of Notches induced in Canton-S +. Therefore, the proximity of N^+ to heterochromatin, as in w^{m4} and in rst^3, increases the likelihood that a Notch deficiency will survive as a heterozygote, and does not reflect any position effect on mutability. Such an increase in survival must result from the fact that a greater number of Notch deficiencies which lack fewer than 50 euchromatic bands are produced in w^{m4} and rst^3 than in +. Proximity of N^+ to heterochromatin would not of itself produce more such deficiencies if there were some physical limitations so that X-ray induced deficiencies could never exceed a distance equivalent to 50 bands. The greater number of Notch mutants induced in the inversion stocks clearly implies that numerous deficiencies of N^+ have a total extent exceeding a distance equal to 50 bands, but a sufficient proportion of that total extent is heterochromatin, thus restricting the euchromatic loss to less than 50 bands.

Such a view of the effect of the proximity to heterochromatin should apply to any euchromatic locus which by inversion has been brought close to heterochromatin. MULLER (1940) reported the effect of irradiating the scute-8 (sc^8) inversion. In sc^8 the y^+ locus is adjacent to a large segment of heterochromatin. Muller recorded 73 yellow mutants among 22,330 F_1 females following irradiation of sc^8 males with 4000 r, which is at least three times as many as would be expected from a similar irradiation of non-inverted X-chromosomes. SIDOROV (1936) performed a similar study on sc^8 and noted that of 73 fertile yellow mutants, only 11 were male-viable. Of these 11, moreover, nine were yellow-achaete mutants; that is, not effects at the y^+ locus alone but involving the adjacent ac^+ locus as well. Such a *y ac* mutant is likely a male-viable deficiency, similar to that produced by MULLER (1935), even though SIDOROV considered the *y ac* mutants to be true double mutations. Thus, at the yellow locus, like the Notch, proximity to heterochromatin facilitates deletion because

of the " inertness " of heterochromatin. In addition, BELGOVSKY and MULLER (1938) noted numerous changes at the forked (*f*) locus following irradiation of the B^{M2} inversion.

The fact that detectable deletions are more numerous following irradiation of the inversion stocks further implies that a considerable number of deletions too long to survive as heterozygotes are produced following irradiation of normal chromosomes, even though they are ordinarily undetectable. Interestingly enough, one of the Notch mutants produced from Canton-S + in this experiment was just such an excessively long deficiency which was detected because the deleted material was simultaneously inserted in the third chromosome. Examination of the salivary chromosomes showed that the X-chromosome is deficient from approximately band 1E2 to 3C8. This Notch deficiency (N^{50k11}), extending some 70 bands, does not survive unless accompanied by the insertion, which itself is slightly shorter than the deficiency. DEMEREC (1940) described some insertional translocations of the white-Notch region which were even longer. Thus, the production of dominant lethal deficiencies incapable of surviving in heterozygotes is a normally occurring, and probably not infrequent, consequence of irradiation.

DEMEREC and FANO (1941) considered that Notch deficiencies less than about 15 bands (counting doublets as one band) in length resulted from one-hit effects; longer ones from two-hit effects. If this is true, then Notches produced in w^{m4} and rst^3 should show a non-linear relationship with dose since the great majority of them must be deficient for more than 15 bands. On the other hand, MULLER (1940) found that yellow mutants produced from sc^8 (an analogous situation) followed a linear rather than an exponential relationship with dose. Therefore, either the spread of effect from one hit commonly exceeds the limit of 15 bands conceived by DEMEREC and FANO, or else an excessive spread of effect is characteristic of heterochromatin. In future experiments it would be desirable to determine the length of the heterochromatic loss in the case of mutants induced in w^{m4} and rst^3 as well as to use other doses than 5000 r in order to distinguish between one- and two-hit effects.

The failure to produce any male-viable split mutations in the present experiment may seem surprising. Not one was detected among nearly 53,000 F_1 females heterozygous for *spl* while 174 Notch mutants were being found. This significant failure of X-rays to induce *spl* mutants suggests two things: a) split mutants arise only from true non-lethal gene changes affecting the locus whose loss gives rise to Notch, and b) X-rays are incapable of inducing true gene mutations at the spl^+ locus. Split, then, may be considered as an allele of Notch in the sense that every Notch is a deficiency of the spl^+ locus.

DEMEREC (1934) failed to induce any male-viable facet mutants although 27 Notch mutants (deficiencies of *fa*) were found. Thus, the relations of spl^+ to Notch very likely are duplicated by fa^+. All tested *N* mutants uncover both *spl* and *fa*, and X-rays may well be capable of producing neither male-viable *spl* nor male-viable *fa* mutants. It is improbable that a unitary N^+ locus exists,

although use of the symbol is convenient. The wild-type condition is better indicated as *spl*$^+$ *fa*$^+$, as suggested by BAUER (1943).

Can it be true that X-rays produce no true gene mutations at any locus in *Drosophila* (see LEFEVRE 1950)? Many male-viable mutants are found following irradiation, such as the whites in the present experiment. These viable white mutants are rarely associated with cytological abnormality, whereas lethal whites are nearly always detectably deficient (see DEMEREC 1941, and descriptions in BRIDGES and BREHME 1944). There are few, if any, "no-band" lethal whites. It is the viable whites which correspond to the cytologically normal lethal Notches. Reasons have been presented for believing that these Notches, like the detectably deficient Notches, result from real, though invisibly small, loss. The X-ray-induced male-viable whites may well result from the same cause. PANSHIN (1938) has reported the survival of a homozygous white deficiency; deficiency of yellow, too, is not necessarily lethal, according to MULLER (1935). Two categories of gene loci may be visualized. Some loci, represented by w^+ and y^+, are dispensable and may survive as deficiencies if neighboring loci are not simultaneously deleted. Other loci, represented by *spl*$^+$, are essential and any loss, however small, is lethal. Viable mutants at loci such as *spl*$^+$ arise only by true gene mutation, but true gene mutation is not produced by X-radiation.

SUMMARY

Following irradiation of Canton-S +, Abruptex, white-mottled-4 and roughest-3 males with 5000 r, white and Notch mutations were detected in the F_1 females. Approximately 25,000 F_1 females were examined in each series. The incidence of both white and Notch mutants produced in *Ax* was very similar to that observed in Canton-S +. Thus, the extra band in *Ax* does not contain a duplicated N^+ locus, and the Abruptex phenotype must result from a position effect. About 1/7 of all Notches simultaneously involve the white locus.

Both white and Notch effects were greatly increased when the inverted X-chromosomes were irradiated. In w^{m4} and rst^3 the N^+ locus lies close to proximal heterochromatin. The frequency of Notches induced in w^{m4} was similar to that in rst^3; being more than three times greater than the Notch frequency in the non-inverted X-chromosomes. In w^{m4} the excess number of Notches results from deficiencies extending into the heterochromatin. Therefore, proximity to heterochromatin facilitates the production of deletions of euchromatic loci viable as heterozygotes. In normal X-chromosomes Notch deficiencies longer than 50 salivary bands are not detected because such long deficiencies are dominant lethals. However, when the bulk of deleted material is heterochromatin, much longer deficiencies can survive in heterozygotes. It is not clear whether these long deficiencies result from one-hit or two-hit events.

Male-viable split mutations could have been detected in the w^{m4} and rst^3 irradiations. Nearly 53,000 F_1 females heterozygous for *spl* were examined, and while 174 Notch mutations were found, not one male-viable split mutation

was observed. The failure of X-rays to induce *spl* alleles suggests that X-rays do not give rise to true gene mutations but only to destructive changes. All Notch mutants likely result from deletion, even though many show no cytological loss. A *spl* mutant, however, must result from true mutation of the spl^+ locus, loss of which produces Notch.

In Canton-S + and *Ax* the total number of white mutants (viable and lethal) closely approximated the total number of Notch mutants. It may be suspected that male-viable white mutants are equivalent to the " no-band " Notches, and those induced by X-rays really result from genetic destruction rather than from true mutation. The loss of some loci, such as w^+ and y^+, is not necessarily lethal if the deletion does not include adjacent loci. The loss of other loci, such as spl^+, is invariably lethal.

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GENETIC DETERMINATION OF TYROSINASE THERMOSTABILITY IN NEUROSPORA[1,2]

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IT was shown in a previous study that the tyrosinase activity of wild strain 4A of *Neurospora crassa* is influenced by the temperature of cultivation (HOROWITZ and SHEN 1952). The strain produces almost no tyrosinase activity when cultured at 35° C on a medium which favors the production of strong activity in 25° cultures. The evidence indicated that the temperature effect is not due to formation of a tyrosinase inhibitor, but to a net decrease in tyrosinase synthesis at the higher temperature. Attention has been called to the resemblance between this case and that of the Himalayan rabbit, in which the formation of melanin, the end-product of tyrosinase activity, fails to take place at body temperature (HOROWITZ 1951).

On the basis of the analogy with Himalayan, a search was made for strains in which tyrosinase is produced at both 25° and 35° (analogous with self-colored) and strains in which the enzyme is produced at neither temperature (analogous with albino). A number of strains analogous with self-colored, in the above sense, were found. When the tyrosinase in crude extracts of one of these strains was tested, it was found to be much more stable to heat than is the tyrosinase obtained from strain 4A. This discovery led us to an investigation of the following points: (a) the mode of inheritance of the thermostability, (b) the chemical basis of the difference in thermostability, and (c) the relationship between thermostability and enzyme production at 35°.

THE STRAINS

We distinguish no wild type of *N. crassa* with respect to tyrosinase thermostability, since among strains collected in nature it appears that some (referred to as T^S strains) produce the thermostable tyrosinase and others (T^L strains) the thermolabile tyrosinase. Of the original wild stocks obtained from various sources by BEADLE and TATUM (1945) and since maintained by serial transfer, one—Abbott 4A—is of the T^L type, and two others—12a and 25a—are T^S strains. Abbott 4A appears in the ancestry of all stocks which we have determined to be of the T^L type, and, similarly, the T^S character can be traced back to 12a and 25a. Neither character has ever been observed to arise by mutation in our stocks, but this does not exclude the possibility that they may do so.

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Strain 4A has been used as representative of the T^L type in many of the experiments to be described. Since asexually propagated lines of Neurospora may in time become heterocaryotic for heterogeneous assortments of genes through spontaneous mutation, we have repeated all essential tests with a recently isolated single-ascospore culture of type T^L, designated No. 4-137.

As representative of type T^S, strain No. 854a, containing both 12a and 25a in its ancestry, and a recent derivative of it, No. 4-121, were used. Inasmuch as strains of a given type gave identical results in all essential respects regardless of whether they were new isolates or old stocks, we shall dispense with the use of stock numbers in what follows and refer to the strains by the symbols T^S and T^L.

METHODS

The measurement of tyrosinase activity

Tyrosinase activity was determined colorimetrically and manometrically by the procedures previously described (HOROWITZ and SHEN 1952).

In the colorimetric method, the production of 2-carboxy-2,3-dihydroindole-5,6-quinone (hallachrome) is measured under standard conditions. This substance, a red pigment, is the first colored intermediate formed in the oxidation of tyrosine and 3,4-dihydroxyphenylalanine (dopa) by tyrosinase. It undergoes a base-catalyzed (non-enzymatic) decarboxylation and rearrangement, and subsequently an enzymatic oxidation, giving rise eventually to the precursor of melanin (RAPER 1932; MASON 1948). The decarboxylative rearrangement is the rate-limiting step in melanin formation (MASON and WRIGHT 1949), and at pH 6, where we have carried out all activity measurements, hallachrome is relatively stable (RAPER and WORMALL 1925). This property makes it possible to use the rate of hallachrome production as a measure of tyrosinase activity. It was shown by HOROWITZ and SHEN that the maximum rate of hallachrome production is proportional to the enzyme concentration in Neurospora extracts.

Because of its convenience, the colorimetric method is especially suitable for studies such as this one in which tyrosinase assays are carried out in large numbers. It has the additional advantage of good sensitivity (5 to 10 times more sensitive than the manometric assay), making it possible to test crude extracts at high dilutions and thereby minimize the possibility of interference by extraneous substances. In view of the general lack of uniformity among workers with regard to tyrosinase assay procedures, however, and because of the complexity of the tyrosine-tyrosinase reaction, we thought it desirable to check the colorimetric results by an independent method. For this purpose we have measured oxygen consumption manometrically in a number of experiments. The results obtained by the two methods are in good agreement, as shown in tables 1 and 4 below.

Cultures were grown for 4 or 5 days at 25° unless otherwise indicated, on the low sulfur liquid medium described by HOROWITZ and SHEN. The mycelium was washed with distilled water, weighed, and ground in a mortar with

sand and 20 ml of cold 0.1 M phosphate buffer, pH 6, per gram wet weight of mycelium. The resulting suspension was centrifuged to remove sand and debris. The supernatant, henceforth referred to as "crude extract," was used as a source of the enzyme in the early experiments. The more recent work has been carried out with a purified preparation to be described later.

Colorimetric determinations of tyrosinase activity were made with a Klett-Summerson photoelectric colorimeter, using filter No. 42. A measured quantity (0.5 ml or less) of the enzyme preparation was placed in a colorimeter tube, followed by sufficient 0.1 M phosphate, pH 6, to bring the volume to 4.5 ml. After temperature equilibration in a 30° water bath 0.5 ml of M/60 L-tyrosine or L-dopa (or M/30 DL-dopa) was added. Readings were taken at 5-minute intervals. Aeration was accomplished by shaking. Colorimetric rates as reported here are equal to the maximum increment in optical density (expressed as colorimeter units, *c.u.*) obtained in a 5-minute interval. With dopa as substrate for the purified enzyme, the maximum rate is attained in the first 5 minutes; with tyrosine there is a lag of up to 20 minutes, depending on the concentration of the enzyme. When the crude enzyme is used there may also be a brief lag with dopa.

Manometric measurements were made with the Warburg apparatus at 34.5°. Two ml of the enzyme solution were used in each vessel, with 0.25 ml of tyrosine or dopa solution in the sidearm, and alkali in the well. Manometric rates are given as the maximum oxygen consumption, in microliters, obtained in any 5-minute period.

It should be noted that dopa is stable at pH 6, so that the corrections for spontaneous oxidation which are necessary in experiments run at pH 7 or higher do not apply here.

Thermal inactivation

Most of the heat-inactivation experiments were carried out in the 54° to 60° temperature range, and for this purpose a thermostatically controlled water bath constant to ± 0.4° was used. The fluctuations in bath temperature are the source of the largest error in these experiments, since the temperature coefficient of inactivation is high in this temperature range.

Two different procedures have been employed in experiments where it was desired to measure the rate of thermal inactivation. In the first, 0.5-ml portions of the enzyme solution were measured into a series of 15-ml test tubes held in a rack. The tubes were stoppered, and at zero time the rack was immersed in the water bath. Tubes were removed at intervals and rapidly chilled in ice. The samples were diluted with buffer before assaying. In the second procedure, a single 50-ml tube containing a relatively large volume of buffer was brought to temperature equilibrium in the water bath, and a small volume of enzyme solution was run in at zero time. Samples were removed at intervals and chilled. The second method is the more accurate of the two, although both have given satisfactory results.

EXPERIMENTAL RESULTS

Thermostability of the enzymes

Table 1 shows the results of an experiment in which crude extracts of T^S and T^L were heated at 58° for varying periods of time and were then assayed for tyrosinase activity manometrically and colorimetrically, using dopa as substrate. The results obtained by the two assay methods are in good quantitative agreement and show that the tyrosinase activity from strain T^S is considerably more stable at 58° than is the activity from T^L, roughly 16 times more stable in this experiment.

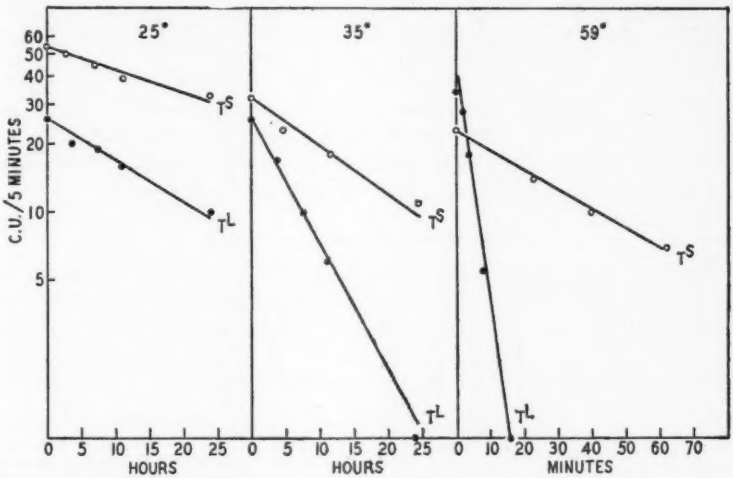

FIGURE 1.—Stability of T^S and T^L tyrosinases at three temperatures. Ordinate: residual activity in colorimeter units per 5 minutes, plotted on a logarithmic scale. Abscissae: time of exposure to the indicated temperature.

In figure 1 are plotted semilogarithmically the data from a series of experiments in which the stabilities of T^S and T^L tyrosinases were compared at three temperatures. The assays were colorimetric, with tyrosine as substrate. The data fall on a series of straight lines, within the limits of experimental error, indicating that the enzymes are inactivated in a first order process, i.e., a reaction whose rate is proportional to the tyrosinase concentration. The data of figure 1 can be summarized in terms of the half-lives of the enzymes at the given temperatures, as shown in table 2. Further consideration of the inactivation reaction will be postponed until after presentation of the genetical part of the investigation.

In other experiments, we have compared the T^S and T^L enzymes with respect to their pH optima and sensitivity to inhibition by cysteine. Although the possibility of small differences is not excluded by the experiments, no large

TABLE 1

Stability of T^S and T^L tyrosinases at 58°.

Each colorimeter tube contained 0.5 ml heat-treated crude extract + 4 ml buffer + 0.5 ml DL-dopa. Each Warburg vessel contained 1 ml heat-treated crude extract + 1 ml buffer + 0.25 ml L-dopa. c.u. = colorimeter units.

Strain	Minutes at 58°	Maximum rate			
		c.u. per 5 minutes	Percent	μl O_2 per 5 minutes	Percent
T^S	0	185	100	34.1	100
"	40	158	85	29.2	86
"	80	117	63	23.6	69
T^L	0	113	100	29.5	100
"	5	79	70	20.4	69
"	10	30	27	6.4	22
"	20	7	6.2	3.0	10

differences comparable to the thermostability difference were found. Both enzymes have optima in the neighborhood of pH 7 (with tyrosine as substrate), and both enzymes are completely inhibited by 2×10^{-4} M cysteine.

The genetics of tyrosinase stability

The inheritance of tyrosinase stability and lability was studied in the crosses $T^S \times T^L$, $T^S \times T^S$ and $T^L \times T^L$. Ascospores were isolated in order, and one member of each spore pair was tested for tyrosinase stability. Each spore isolate to be tested was cultured for 5 days at 25° on the low sulfur medium, and a crude extract was prepared from the mycelium. The extract was divided into

TABLE 2

Half-lives at three temperatures.

Temperature	Half-life (hours)	
	T^S	T^L
25°	29	16.5
35°	15.5	5.5
59°	0.5	0.05

two portions, one of which was incubated at 59° for 30 minutes while the other, serving as a control, remained at 0°. Both samples were then tested for tyrosinase activity, using tyrosine as substrate. The heat treatment was sufficient to destroy the labile enzyme, but not the stable enzyme. The results were thus essentially qualitative in nature and could be read by inspection. Nevertheless, colorimetric measurements were routinely made in order to make it possible to detect intermediate grades of stability. No clear intergrades were noted. The results are therefore presented in table 3 on a qualitative basis.

Three different crosses of $T^S \times T^L$ were made, involving different pairs of parents. A number of the progeny from one of the crosses showed very weak or no tyrosinase activity in the unheated control sample, and the thermosta-

bility could not be determined. Where this occurred the ascus is designated as incomplete. Similar negative progeny were obtained in some asci from the cross $T^S \times T^S$. A number of negative spores from the $T^S \times T^L$ cross were retested after having grown for 7 days instead of the standard 5, and they were then found to show some activity. The fact that negative spores occur in only certain crosses suggests that genes governing the time of development of tyrosinase activity may be segregating here.

TABLE 3

The inheritance of tyrosinase stability and lability.

S = good activity after 30 minutes at 59°. L = no activity after 30 minutes at 59°. − = no activity in unheated control.

Cross	Complete asci	Number	Incomplete asci	Number
$T^S \times T^L$	SSLL	3		
	LLSS	4		
	SLSL	4	None	
	LSLS	5		
	SLLS	2		
	LSSL	2		
$T^S \times T^L$	SSLL	5		
	LLSS	2		
	SLSL	1	None	
	LSLS	3		
	SLLS	0		
	LSSL	5		
$T^S \times T^L$	SSLL	4	SS-L	1
	LLSS	1	SSL-	1
	SLSL	1	L-SS	1
	LSLS	1	SLS-	1
	SLLS	2	S-LS	1
	LSSL	3	-SSL	2
	Total	48		7
	Grand Total	55, with 33 MII segregations.		
$T^S \times T^S$	SSSS	4	SSS-	2
			SS-S	1
			S-SS	1
			-SSS	2
			--SS	3
			-SS-	2
			---S	1
$T^L \times T^L$	LLLL	4	None	

A total of 48 complete and 7 incomplete asci were analyzed from the $T^S \times T^L$ crosses. All of the complete asci showed a 1 : 1 segregation for stability *vs.* lability, and the incomplete asci are all consistent with a 1 : 1 segregation. It thus appears that lability and stability are determined by a pair of alleles. The locus is at least 30 standard map units from its centromere (60 percent second-division segregations). Its linkage group is not known and may be difficult to determine, since the locus segregates almost at random with respect

to the centromere (complete randomness gives 67 percent second-division segregations).

It is proposed that the symbols T^S and T^L be used to designate these alleles, where T symbolizes *tyrosinase* and the superscripts designate *stable* and *labile*, respectively.

The chemical basis of stability and lability

We now take up the question of whether the difference in tyrosinase stability is based on a structural dissimilarity in the tyrosinases, or whether it can be accounted for on the basis of identical enzymes with differences in the production of a destructive or protective agent. It has already been shown that the inactivation is first order with respect to the enzyme, a fact which permits us to conclude only that the reaction is either monomolecular or pseudomonomolecular. By " pseudomonomolecular " reactions are meant bimolecular reactions which resemble a monomolecular process kinetically due to the fact that the concentration of one reactant does not change. The constant reactant can be a catalyst, or it can be a substance which is present in great excess. Since none of these possibilities has been excluded it is evident that the data up to this point do not give much information with respect to the mechanism of the inactivation. We have carried out three kinds of experiments in order to throw light on this problem: partial purification of the enzymes, dilution experiments, and mixing experiments. We shall consider these in order.

Purification of the enzymes.—A partial purification of the enzymes was effected by the procedure to be described. All operations were carried out at or near 0°. The course of the purification was followed by measuring activity of the fractions per unit of protein nitrogen. The latter was determined by micro-Kjeldahl digestion of 5 percent trichloroacetic acid precipitates, followed by Nesslerization.

Cultures were grown for 5 days at 25° in Fernbach flasks containing 200 ml of the low sulfur medium. A crude extract was prepared, using 3 ml of phosphate buffer per gram wet weight of mycelium. After centrifugation, the insoluble material was extracted again with the same volume of buffer and the two extracts pooled.

The pH was adjusted to pH 7 to 7.2 with 1 *N* NaOH, and manganous sulfate was added in the ratio of 1 ml of a 1 M solution per 20 ml of extract. The pH was brought back to pH 7.2 with NaOH and the precipitate removed by centrifugation. The precipitate was washed with a small volume of water and the washing added to the supernatant. This treatment removes the turbidity from the crude extract and leaves the tyrosinase activity in the clear supernatant.

Solid ammonium sulfate was added to the cold solution to 80 percent saturation (at 0°) and the precipitate, containing the enzyme, was centrifuged down and dissolved in 1 to 2 ml of water per gram of the original mycelium.

One volume of ice-cold acetone was slowly added to the aqueous solution containing the enzyme; the precipitate was centrifuged down and discarded.

More acetone was added to bring the concentration to 60 percent by volume, the precipitate was collected by centrifuging and was triturated with two successive small volumes (5 to 10 ml) of phosphate buffer (M/10, pH 6). The insoluble material was discarded.

The enzyme was reprecipitated from solution by the addition of 4 volumes of a saturated solution of ammonium sulfate, and it was again dissolved in a small volume of buffer. The clear, colorless solution was then dialyzed against phosphate buffer in the refrigerator.

The dialyzed solution constituted the partially purified enzyme preparation. In table 4 the activities of the crude and purified enzymes are compared. It can be seen that a 42- to 46-fold purification of the stable enzyme was obtained, but only a 12- to 14-fold purification of the labile enzyme, although identical fractionation procedures were employed in the two cases. The difference is attrib-

TABLE 4

Activity of crude and partially purified Neurospora tyrosinases.

Q_{O_2} = μl O_2/mg protein/hour. Protein obtained by multiplying the trichloroacetic acid precipitable nitrogen by the factor 6.25.

Strain	Preparation	Substrate	Activity		
			c.u./yN/5 min.	μl. O_2/yN/5 min.	Q_{O_2}
T^S	Crude	DL-dopa	1.94		
	"	L-tyrosine	0.99		
	Purified	DL-dopa	89.3		
	"	L-dopa		7.68	14,800
	"	L-tyrosine	41.8	7.43	13,700
T^L	Crude	DL-dopa	3.46		
	"	L-tyrosine	1.30		
	Purified	DL-dopa	40.0		
	"	L-dopa		3.20	6,140
	"	L-tyrosine	17.5	3.69	7,080

utable to a large loss in activity of the labile enzyme in the acetone step. The over-all recovery of the stable enzyme was 28 percent, and of the labile enzyme 7 percent.

Table 4 shows additionally that the two enzymes do not differ from one another significantly in their relative activities toward tyrosine and dopa. The two substrates are oxidized at nearly equal rates manometrically, but dopa is converted into hallachrome more than twice as fast as is tyrosine. This is consistent with the fact that an extra atom of oxygen is required in the oxidation of tyrosine. It can also be seen that no significant change in the relative reactivities of the two substrates resulted from the purification. This point is of some interest in view of the fact that several authors have noted an increase in diphenolase activity relative to monophenolase activity in the course of the purification of mushroom tyrosinase (KEILIN and MANN 1938; MALLETTE *et al.* 1948).

Finally, table 4 further illustrates two technical points mentioned previously: the proportionality between colorimetric and manometric rates when determined with the same substrate, and the greater sensitivity of the colorimetric method. The smallest detectable increment in volume of oxygen with the usual Warburg equipment (vessel constants around 1.5) is 0.75 μl, and the smallest detectable increment in optical density with the Klett-Summerson colorimeter is one colorimeter unit. But, as shown in the table, an amount of enzyme which in 5 minutes catalyzes the uptake by dopa of a quantity of oxygen equal to 10 times the minimal amount detectable, will in the same period of time produce a change in optical density equal to 90 times the minimal reading. With tyrosine as substrate the sensitivity difference is appreciably smaller.

Thermostability of the partially purified enzymes.—The stabilities of the partially purified enzymes were compared at 59°. The semi-log plots are shown in figure 2. The half-life of the T^L enzyme is 3 to 4 minutes, a value which does

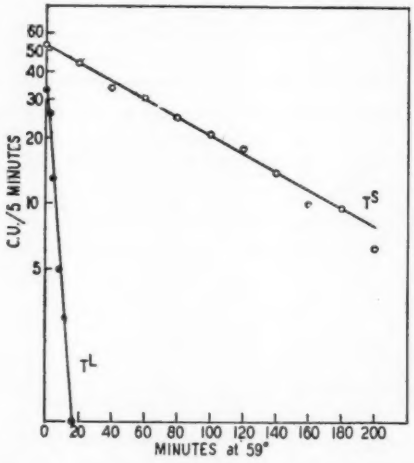

FIGURE 2.—Stability of the partially purified tyrosinases at 59°. Substrate: DL-dopa.

not differ from that obtained with the crude extract. The half-life of the purified T^S enzyme is approximately 70 minutes, a figure significantly greater than that found for the crude enzyme. It appears that purification of the tyrosinases accentuates rather than diminishes the difference in thermostability.

Dilution experiments.—In these experiments, enzyme stability was measured at various dilutions of the two enzymes. The interpretation rests on the following deductions from chemical kinetics: 1. If inactivation of the enzyme is monomolecular, the stability will be independent of concentration, and the fraction of the original activity remaining after time t will equal e^{-kt}, where k is a constant. 2. If the inactivation is pseudomonomolecular, the stability will increase with increasing dilution; the fraction remaining at time t will be given

by $e^{-k'tb}$, where b is the concentration of the second reactant (tyrosinase-destroying agent in this case). A special case is that in which the inactivation results from a reaction between the enzyme and the solvent. In this event, b will not change with dilution, and the reaction will therefore appear to be monomolecular by the dilution test. But since the solvent (phosphate buffer) is the same for both enzymes, this result will indicate a structural difference in the two enzymes, regardless of the actual mechanism of the inactivation.

In table 5 are shown the results of stability measurements made over a 32-fold range of enzyme concentrations. In each case, the indicated quantity of purified enzyme solution was diluted to the stated volume with buffer in a colorimeter tube. The tubes were heated in the water bath, cooled, and assayed with DL-dopa as substrate. The results show that dilution of the enzymes is without significant effect on their stability. If the reaction were pseudomono-

TABLE 5

Effect of dilution on the inactivation of T^S and T^L tyrosinases.

Strain	Ml enzyme	Final vol., ml	Inactivation temperature	Exposure time, minutes	Residual activity	
					c.u./5 min	Percent
T^S	0.5	4.5	59°	0	43	100
	2.8	"	"	40	151	63
	1.4	"	"	"	76.5	63
	0.7	"	"	"	33	55
	0.35	"	"	"	18.5	61
	0.175	"	"	"	8.5	57
	0.0875	"	"	"	4	53
T^L	0.2	2.25	54°	0	56	100
	0.8	"	"	8	123	55
	0.4	"	"	"	60	54
	0.2	"	"	"	29	52
	0.1	"	"	"	17	61
	0.05	"	"	"	9	64
	0.025	"	"	"	4.5	64

molecular, each fraction in the last column would approximate to the square root of the value above it, except in the special case discussed earlier. The essential constancy of the values is evidence for dissimilarity of the tyrosinases.

Mixing experiments.—The mixing experiment is based on the expectation that if the difference in tyrosinase stability is caused by differences in the enzymes themselves, then the rate of thermal inactivation of a mixture of the two will equal the sum of the individual rates; whereas if the difference is attributable to unequal concentrations of a destructive agent in the enzyme preparations, then the inactivation rate of a mixture will show an interaction effect.

Table 6 presents an experiment in which quantities of the two enzymes previously adjusted to approximately equal activities were inactivated singly and together at 59°. With the possible exception of one value, there is no significant departure from a simple additive effect. This experiment is thus in

accord with those already described in indicating a qualitative difference in the enzymes produced under the influence of the T^S and T^L alleles.

The second phenol oxidase

In the course of these studies a second phenol oxidase has been discovered in Neurospora. In preliminary experiments with crude preparations it has differed in the following respects from the enzymes described in this paper: it catalyzes a rapid oxidation of dopa, but has little if any effect on tyrosine; it is more thermostable than either of the tyrosinases we have been dealing with; it is produced in considerably larger amounts in 35° cultures than in the

TABLE 6

Inactivation of a mixture of T^S and T^L enzymes.

The indicated mixtures of enzymes and buffer were heated at 59°, then cooled and diluted to 4.5 ml with buffer, followed by 0.5 ml DL-dopa. Values in parentheses are the rates expected in the absence of interaction.

Ml enzyme		Ml	Minutes	Activity
T^S	T^L	buffer	at 59°	c.u./5 min
0.2	0	0.3	0	28
"	"	"	2	27
"	"	"	4	23
"	"	"	8	24
"	"	"	20	22
"	"	"	40	20
0	0.2	"	0	25
"	"	"	2	18
"	"	"	4	10
"	"	"	8	4
"	"	"	20	2
"	"	"	40	1
0.2	0.2	0.1	0	56 (53)
"	"	"	2	45 (45)
"	"	"	4	35 (33)
"	"	"	8	27 (28)
"	"	"	20	21 (24)
"	"	"	40	14 (21)

25° cultures; and it is found chiefly in the medium. Further studies are in progress.

On the basis of its known properties, it is safe to assert that the second phenol oxidase has had no significant disturbing effect in any of the experiments reported above.

Tyrosinase production at 35° in relation to thermostability

As mentioned previously, the existence of tyrosinases differing in thermostability was first discovered in two strains which differed also in their tyrosinase content at 35°. The findings were such as to suggest that the absence of tyrosinase from one of the strains when grown at 35° was directly related to the fact that it produced the thermolabile form of the enzyme. This inference

has not been supported by subsequent developments. The production of tyrosinase at 35° has not been inherited in a simple manner in the crosses so far analyzed. The segregations are not regularly 1 : 1, intermediate grades of expression are frequently obtained, and in several instances the initial classification of a culture has changed when the test was repeated at a later date. The results suggest multifactorial inheritance, with the possibility that uncontrolled physiological factors are important in the expression in some strains. All this is in marked contrast to the thermostability character. A number of attempts to demonstrate cytoplasmic inheritance of the ability to form tyrosinase at 35° have given only negative results.

It is not surprising to find that the enzyme content of a culture can vary independently of the thermostability of the enzyme. The net rate of enzyme production is of course determined by the rates of synthesis and loss. Thermal inactivation is but one of several processes which can contribute to the total rate of enzyme dissipation, and the extent to which thermal destruction influences the quantity of enzyme actually present in the cell cannot be predicted

TABLE 7

Phosphate-induced tyrosinase production at 35° in strain 4A.

The cultures were 6 days old. The heat treatment consisted of 30 minutes at 59°.

Phosphate concentration	Incubation temperature	Wet wt. of mycelium	c.u./0.1 ml extract/5 minutes			
			Untreated extract		Heat-treated extract	
			Tyr.	Dopa	Tyr.	Dopa
0.0074 M	25°	1.0 gm	18	19	0	1
"	35°	0.39 gm	0.1	2		1.4
0.3 M	35°	0.42 gm	60	106	0	12

without specifying the magnitude of the other parameters. Similar ideas have been embodied by DANNEEL (1941) in a hypothesis to account for the Himalayan color pattern in rabbits, and by ALLEN (1950) in a theory of thermophily in bacteria.

Experimental support of the above interpretation comes from the finding that tyrosinase production at 35° can be induced in strain 4A by nutritional means. Strain 4A is characterized by little or no tyrosinase production at 35° on the low sulfur medium, whereas at 25° it forms the thermolabile tyrosinase, as shown in this paper. When the medium is made 0.3 M in phosphate, however, 4A produces considerable activity at 35° (table 7). The enzyme is still of the thermolabile type, as shown in the table. (The thermostable fraction of the activity observed with dopa as substrate is due to the presence of a small amount of the second phenol oxidase.) Other experiments have shown that the half-life of the phosphate-induced enzyme at 59° is no different from that of the tyrosinase produced at 25° on unsupplemented medium.

It should be noted that owing to the high phosphate concentration which must be employed to obtain the above effect, the possibility remains open that the active substance is not phosphate, but a trace impurity.

DISCUSSION

Since all of the tests described above have failed to disclose the presence of any substance in the tyrosinase preparations capable of producing the observed difference in stability, we conclude that a structural dissimilarity, determined by the alleles T^S and T^L, exists between the two enzymes. This case thus provides direct evidence of the qualitative alteration of an enzyme as a result of gene substitution.

MAAS and DAVIS (1952) have recently presented evidence for the production of an abnormally thermolabile enzyme in a mutant of *E. coli* characterized by a requirement for pantothenic acid at temperatures above 30°. The enzyme concerned is that which couples β-alanine with pantoic acid to form pantothenic acid. This case appears to be similar to ours in principle, although it was not possible for the authors to carry out a genetic analysis in their strain of *E. coli,* a circumstance which is especially regrettable in view of the unusual origin of the temperature mutant (apparently spontaneously from a mutant requiring pantothenate at all temperatures).

In a series of investigations by PAULING, ITANO and collaborators (PAULING *et al.* 1949; ITANO and NEEL 1950; ITANO 1951) several electrophoretically abnormal forms of hemoglobin have been found in human patients. The abnormal hemoglobins appear to be inherited in a simple way, although only in the case of sickle-cell anemia have sufficient familial data been accumulated to make a definite genetic conclusion possible. The evidence shows that sickle-cell anemia is inherited as an incompletely recessive gene (NEEL 1949). Sickle-cell hemoglobin differs from normal in solubility as well as electrophoretically (PERUTZ and MITCHISON 1950).

Two alternative interpretations of these findings will be considered. The first of these, which we can call the template modification hypothesis, assumes that the specific molecular structure of each protein is copied from a template. Each template is considered to be associated with a particular gene, and gene mutation can result in a qualitatively new kind of template. The modified template will produce a correspondingly modified protein. This theory readily accounts for all of the observations, and it has much to recommend it, including the fact that it predicted the production of structurally altered proteins as a consequence of gene substitution.

The alternative, or rate modification hypothesis, assumes no particular mechanism of protein synthesis, but postulates that once synthesized, the protein undergoes secondary structural modifications which alter its stability, solubility, electrophoretic mobility, or other properties without destroying its essential specificity. These changes may result from limited hydrolysis, oxidation or the like, and are assumed to be controlled by certain genes. Mutation or substitution of one of these genes can, by blocking or accelerating one of the postulated reactions, cause the cell to accumulate a form of the protein which would otherwise be present only in traces. The model of this interpretation, as BONNER (1951) has pointed out, is provided by the metabolism of small

molecules, where it is known that mutations can result in the blocking of individual reactions and the accumulation of intermediates or their derivatives behind the block.

The rate modification hypothesis is not inconsistent with the template theory of protein synthesis, but it is important in template considerations because it provides an alternative explanation of findings which have been regarded as furnishing experimental support of the template idea. The immediate question is whether it is possible to design an experiment which would distinguish between the template modification and the rate modification hypotheses.

A desirable extension of the existing observations would be an investigation of the possibility of multiple allelism at the loci in question. If it can be shown that three or more alleles, each determining a structurally different protein, exist at a locus, the template modification hypothesis would be considerably strengthened, since it would seem most difficult to reconcile such a finding with the notion of mere quantitative increase in a normal cellular constituent. The fact that multiple allelism is known, or at any rate suspected, for a number of genes governing blood cell antigens leads one to expect the same situation will eventually be found among genes governing enzyme synthesis. The difficult question of pseudoallelism becomes critical at this point, however, and it is conceivable that an unambiguous genetic solution to the problem will not be possible by present methods.

SUMMARY

A pair of alleles, T^S and T^L, governing the thermostability of the enzyme tyrosinase, have been found in wild strains of *N. crassa*. Strains carrying T^S produce a tyrosinase characterized by a half-life of at least 30 minutes at 59°, whereas strains carrying T^L produce a tyrosinase with a half-life of 3 to 4 minutes at the same temperature. Allelism between T^S and T^L was inferred from an analysis of 55 asci from the cross $T^S \times T^L$. The locus is at least 30 standard map units from its centromere.

The following lines of evidence indicate that the difference in thermostability of the enzymes is based on a structural difference between them: 1. Partial purification of the enzymes does not cause them to become more alike in thermostability; on the contrary, the partially purified enzymes differ more than the crude enzymes. 2. Thermal inactivation of both enzymes occurs in a first order reaction; the slope of the first order inactivation curve does not change with dilution of the enzyme preparations. 3. The rate of thermal inactivation of a mixture of the two enzymes equals the sum of the individual rates.

The thermostability difference does not account for the fact that some strains of Neurospora produce tyrosinase when cultured at a high temperature (35°), whereas others do not.

A second phenol oxidase, differing in several properties from the tyrosinases, has been discovered in Neurospora.

The main results of the investigation are discussed in relation to the template theory of enzyme synthesis.

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THE RELATIVE EFFECTS OF X-RAYS, GAMMA RAYS AND BETA RAYS ON CHROMOSOMAL BREAKAGE IN TRADESCANTIA[1]

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FOR some time it has been generally considered that the production of chromosomal aberrations in Tradescantia and many other materials is essentially independent of the wave length of the exciting radiation in the region shorter than 1.5 A. This view has not been seriously questioned in recent years and to date has remained one of the fundamental tenets of radiobiology.

A number of isolated instances have been noted in this laboratory in which the effects of 250 kvp X-rays and mixed 1.17 and 1.33 Mev γ-rays from Co^{60} did not conform to this expected biological equivalence. In the course of a series of experiments on the relative effects of Co^{60} γ-rays and P^{32} β-rays on Tradescantia, we have extended our measurements to include a comparison in the X-ray region. A considerable difference between the effects of X-rays and Co^{60} γ-rays or P^{32} β-rays has been observed.

In quantitative radiobiological work of this type, precise and reliable physical dosimetry is a major requirement. For this reason the physical methods used have been described in somewhat more detail than is customary. Due in large part to the continuing program carried out in this laboratory for the past three years on the standardization and calibration of our X-, γ- and β-ray sources, any errors in our absolute radiation measurements should be less than 5 percent.

EXPERIMENTAL METHODS

Biological technique

Tradescantia paludosa (clone 5 of SAX) was used as a source of material in both the pollen tube and microspore experiments. In all the pollen-tube work, the material was collected several hours before irradiation, thoroughly mixed, and desiccated over Drierite for approximately one hour before use to facilitate handling. Storage before desiccation was in glass vials at room temperature.

Temperatures during treatment were maintained within 1 or 2 degrees for the different irradiations. Immediately following irradiation the dry pollen was sowed on slides coated with a lactose-agar medium including colchicine after the method of BISHOP (1949). The slides were cultured in moist boxes at 20°C for 17 to 20 hours and finally fixed in 3 : 1 alcohol-acetic acid. Permanent

[1] Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

slides were made after staining by a modification of the Feulgen technique. In the microspore studies, slides were made four days following irradiation of the inflorescences. Acetocarmine-stained smears were prepared in these cases.

From four to six slides were prepared and usually three or four of these were scored for each dose point in all experiments. All slides in both pollen-tube and microspore studies were scored under code. With the single exception of one microspore experiment, the scoring was carried out by one observer (D. S. DANIELS). Spot checks of the slides by other persons were made from time to time and in no instances were any significant variations in scoring noted, with the exception of one or two of the highest doses. Only those experiments were scored in which good pollen-tube growth was achieved and in which the general appearance of the chromosomes in metaphase was clear and distinct. This selection was made on the basis of the condition of the slides as a whole for each experiment. The choice was not made by the individual who scored the slides.

Although the response of the material to radiation is consistent within one experiment performed on one day, a large random day-to-day variation in the radiosensitivity of Tradescantia pollen has been observed repeatedly in this laboratory. This finding has been confirmed in the present work. Due to such variation in the material, valid comparisons of the effects of different radiations can be made only on representative samples of pollen collected at the same time on the same day. Each experiment reported in the present studies has been carried out on material collected under these conditions.

Radiation sources and dosimetry

X-rays. All X-ray exposures were made using a General Electric Maxitron 250 machine operated at 250 kvp. The X-ray tube was equipped with a beryllium window, affording little inherent filtration. In the present work additional external filtration of the beam by 3 mm of aluminum or 4 mm of copper backed by 1 mm of aluminum was employed in all irradiations. The approximate half-value layers for the filtered beam were found experimentally to be 0.5 mm of copper in the case of the 3 mm aluminum filter and 3.5 mm of copper for the 4 mm copper plus 1 mm aluminum filter. These values correspond approximately to effective wave lengths of 0.2 and 0.06 A for the different X-ray beams.

Victoreen ionization chambers were used to measure all X-ray dosages. Throughout the course of the work these dosimeters were repeatedly checked against other chambers recently calibrated by the manufacturer, and at the termination of the experiments they were recalibrated at the U. S. Bureau of Standards in terms of a standard air chamber. The instruments used were initially compared with chambers whose response had been measured previously in terms of a standard ionization chamber in this laboratory. These various comparisons and cross checks were at all times internally consistent. Additional evidence supporting the validity of the dose determinations was given

by the constant response of the chambers to a standard Co^{60} radiation field. In view of all these measurements and check calibrations, it is very unlikely that the X-ray dosimetry in absolute units can be in error by more than 5 percent.

Gamma-ray exposures were made in a facility [2] shown schematically in figure 1. The radiation source in this installation consists of approximately 60 curies of Co^{60} emitting mixed gamma rays of 1.17 and 1.33 Mev. Radioactive

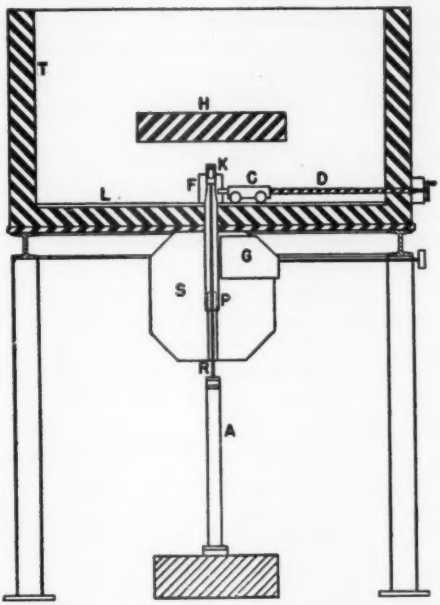

FIGURE 1.—Cobalt gamma-ray irradiation facility.

A—Air cylinder	G—Gate	C—Exposure cart
R—Source push rod	L—Lucite sheet	D—Drive screw
S—Storage shield	F—Lucite collar	H—Ceiling shield
P—Storage position	K—Source capsule	T—Exposure shield

cobalt in the form of thin wafers ½ inch in diameter and ⅛ inch thick is loaded in an aluminum capsule 1½ inches O.D. by 2½ inches long, which is in turn mounted on the end of a pneumatically controlled steel rod. The source is normally stored in a heavy-walled lead safe (S) positioned directly beneath a large tub (T) of hexagonal cross section with 4 inches of lead shielding on

[2] No previous description of this installation exists in the open literature. The design and construction of the facility were carried out largely through the efforts of P. W. Hembree and G. E. Stapleton of this laboratory. It has now been in successful routine operation for several years.

bottom and sides. This upper shield, in which all exposures are carried out, is 5 feet in diameter, has walls 2 feet high and is open at the top. It is supported 5 feet above floor level on heavy steel columns permanently anchored in concrete. No radiation hazard exists in the exposure facility when the source is in its storage safe and the gate (G) is closed. In order to make a radiation exposure, the gate is first opened and the source is raised to operating position through a hole in the bottom of the upper shield by means of the pneumatically driven push rod (R). In this position the source is centered 4 inches above the shield floor by means of a lucite collar (C) through which the aluminum capsule partially projects. The floor of the exposure shield is covered by ½ inch of lucite to eliminate photoelectrons from the lead. The assembly is completed by a small removable lead shield placed directly above the source in order to reduce back scattering from the ceiling of the room.

A great advantage of a radiation source of this type lies in the fact that once it is calibrated the necessity for continual monitoring of every exposure to radiation is eliminated. A few check measurements from time to time and the known rate of decay of the radioactive cobalt allows the intensity to be known at all times.

The γ-ray source was calibrated in terms of the standard radium roentgen by means of a special thimble ionization chamber inserted through a hole in the side of the lead shield. A detailed description of this chamber and a complete discussion of its standardization and use has been given in a report by DARDEN and SHEPPARD (1951). Intensity measurements were made at a number of points along a line perpendicular to the axis of the source capsule and 5 inches above the lucite floor. In the course of these measurements, large blocks of tissue equivalent material (lucite) placed in close proximity to the chamber were shown to result in a very small increase in ion current readings. The presence of scatterers of high atomic number placed close to the ionization chamber, e.g., lead, was found to increase ion currents by 5 to 10 percent. It was found that this scattered background could be reduced to negligible amounts by interposing a few millimeters of plastic between the scatterer and the ion chamber. These measurements, coupled with the known fact that secondary electrons scattered from high-energy γ-rays are predominantly in the forward direction, indicate that the radiation field is little affected by the presence of extraneous objects. It was also shown that the rotation of the source resulted in no appreciable change in radiation intensity along the calibration axis. In an earlier source nonuniform activity of the cobalt slugs resulted in some azimuthal inhomogeneities in the radiation field. This was corrected in the present loading by the use of more uniformly activated cobalt wafers constrained against rotation within the source capsule. Any possibility of these second-order effects modifying the dosages in the present work was eliminated by exposing pollen only along the calibration axis and with no scatterers in the field. From these considerations a very conservative estimate of the errors in the γ-rays doses is of the order of 3 or 4 percent.

P^{32} beta particles from phosphorus bakelite plaques developed at Oak Ridge National Laboratory (RAPER *et al.* 1951) and now commonly used elsewhere

were used as the source of β radiation in the pollen experiments. Exposures were carried out in the chamber shown in figure 2, which was in turn mounted in a thick-walled lucite box. The positioning of the plaques was accomplished by means of push rods extending through this outer shield. The pollen was spread in a monolayer between two thin (2 mg/cm^2) rubber hydrochloride membranes supported on aluminum hoops as indicated. After the pollen was placed in position the two plaques were pushed into contact with the external surfaces of the membranes forming a sandwich with the pollen in the center. The absorption of the β-particles by the plastic membranes was experimentally determined and found to be approximately 2 percent. The two plaques were

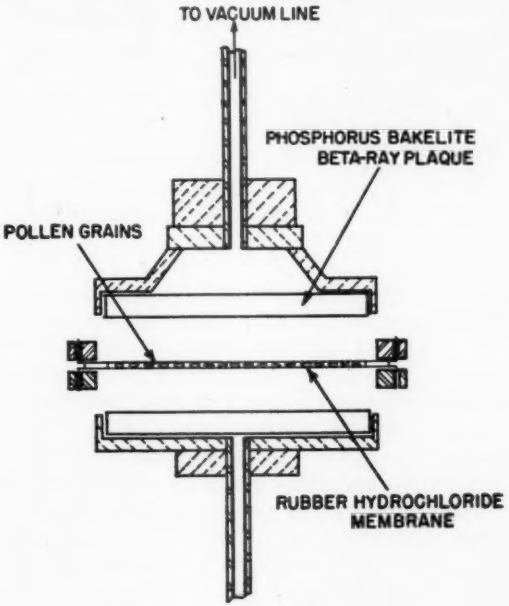

FIGURE 2.—Beta-ray irradiation unit.

selected to be closely identical in activity and each was carefully calibrated by means of a specially designated extrapolation chamber (SHEPPARD and ABELE 1949). By means of these measurements the dose delivered at the surface of the pollen grains was known within 2 or 3 percent. A determination of the depth dose delivered through various plastic materials carried out in this laboratory by DARDEN and SHEPPARD (1952) has shown that, for the P^{32} β-rays used, the absorption in the pollen itself is negligible.

The irradiation chambers used for the X- and γ-irradiations of pollen grains were constructed of lucite with a rubber hydrochloride membrane stretched across the center, as shown in figure 3. As was the case in the β-ray exposures, the pollen was sowed in a monolayer on the membrane surface to insure that

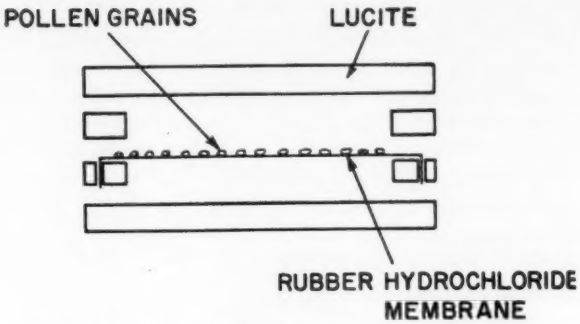

FIGURE 3.—Exposure chamber for X-rays and gamma rays.

all material was in contact with air. In this manner close packing and clumping of pollen, which might possibly lead to local oxygen depletion with consequent change in the radiation response of the material, has been eliminated. After sowing, the pollen adheres to the membranes permitting the chambers to be held in any desired position during exposure.

RESULTS

A summary of the results of all the experiments performed is outlined in tables 1 and 2. Representative dose-aberration frequency curves plotted from these data in the case of the dry pollen irradiations only are shown in figure 4. All doses were measured initially in air in terms of the rep for β-rays and in terms of the roentgen for X-rays and γ-rays, and measurements have been converted to units of energy absorption in tissue for the purpose of comparing the effects of the different agents. The unit of energy absorption in tissue chosen is essentially the energy unit of GRAY (1939) and is taken to be 93 ergs/gram. Normalization of our measured doses in terms of this unit has been accomplished using the tables of LEA (1947), which express energy absorption in water or tissue per roentgen for specific radiation energies. In the absence of any reliable figures for energy absorption in pollen or inflorescences we have assumed the tissues in our experiments to be water equivalent. It is apparent from the tables given by LEA that any errors introduced by this assumption should be less than 1 or 2 percent.

The results of the initial comprehensive experiment, in which the effects of X-, γ- and β-rays on Tradescantia dry pollen were compared in a series of treatments carried out simultaneously on samples drawn from a single homogeneous collection of pollen, are shown in table 1 A and figure 4. Due to practical difficulties in such large-scale experiments involving many irradiations, subsequent comparisons were carried out between only two of the agents at a time. The results of these studies are also summarized in table 1 and figure 4. Throughout this later series of exposures the X- and γ-rays were applied at the same intensities in order to simplify the comparison of the two-hit exchange curves.

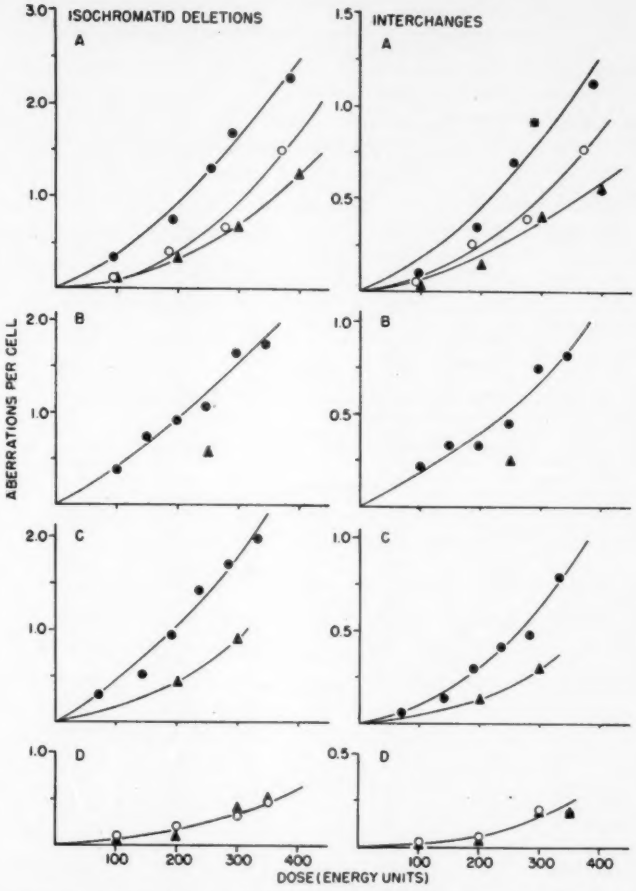

FIGURE 4.—Typical dosage-aberration frequency curves for X-rays, gamma rays and beta rays. ●—X rays; ▲—gamma rays; ○—beta rays.

An outstanding feature of all these experiments is the twofold increase in chromosomal breakage observed for X-rays when compared with γ-rays or P^{32} β-rays; the latter two are approximately equivalent. A further very striking observation in the case of all the isochromatid dose-aberration frequency curves is the definite departure from the generally assumed linear relation. This observation has been previously reported by CATCHESIDE, LEA and THODAY (1946a), but appears to have been overlooked in some quarters in recent years. These points will be discussed in more detail later in this paper.

The surprisingly large difference between the action of X-rays and γ- or β-rays on dry pollen at this point prompted further wave-length studies using

TABLE 1

Comparative effects of X-rays, beta rays and gamma rays on Tradescantia pollen tube chromosomes.

Type of radiation	Dose rate	Measured dose in air	Dose delivered to tissue in units of 93 ergs/gm	Cells		Aberrations per cell		
				Scored	Normal	Chromatid deletions	Isochromatid deletions	Cd/Cd interchanges
			Experiment A					
X	35 r/min	104 r	96	200	113	0.10	0.345	0.095
X		208 r	193	200	53	0.21	0.75	0.345
X		274 r	255	172	12	0.345	1.296	0.698
X		312 r	289	67	1	0.552	1.686	0.925
X		416 r	385	82	0	0.561	2.28	1.134
β	16 rep/min	102 rep	93	200	147	0.065	0.12	0.055
β		204 rep	186	178	73	0.146	0.41	0.253
β		306 rep	278	182	47	0.269	0.665	0.39
β		408 rep	371	167	7	0.317	1.515	0.778
γ	20 r/min	100 r	100	200	161	0.025	0.115	0.035
γ		200 r	200	152	82	0.132	0.342	0.145
γ		300 r	300	190	36	0.168	0.668	0.405
γ		400 r	400	165	13	0.315	1.224	0.558
			Experiment B					
X	21 r/min	107 r	99	200	98	0.09	0.39	0.22
X		160 r	148	200	47	0.18	0.735	0.325
X		213 r	197	200	44	0.125	0.92	0.32
X		266 r	246	200	22	0.19	1.07	0.45
X		320 r	296	150	6	0.273	1.653	0.76
X		372 r	344	150	1	0.44	1.74	0.827
γ	20 r/min	250 r	250	150	56	0.093	0.58	0.253
			Experiment C					
X	20 r/min	77 r	71	100	65	0.08	0.29	0.06
X		153 r	142	100	36	0.10	0.51	0.14
X		204 r	190	150	34	0.07	0.95	0.30
X		255 r	236	75	9	0.28	1.42	0.43
X		306 r	283	50	1	0.32	1.70	0.48
X		357 r	331	125	0	0.36	1.98	0.79
γ	21 r/min	200 r	200	138	62	0.16	0.43	0.13
γ		300 r	300	127	26	0.21	0.905	0.30
			Experiment D					
γ	12 r/min	100 r	100	150	132	0.027	0.066	0.02
γ		200 r	200	100	79	0.06	0.11	0.04
γ		300 r	300	150	66	0.087	0.42	0.20
γ		350 r	350	127	47	0.252	0.496	0.182
β	14 rep/min	110 rep	100	150	130	0.027	0.10	0.027
β		220 rep	200	150	104	0.113	0.213	0.053
β		330 rep	300	150	72	0.173	0.32	0.206
β		385 rep	350	150	65	0.12	0.465	0.173
X	34 r/min	204 r	189	150	56	0.14	0.534	0.246
			Experiment E					
X	35 r/min	105 r	97	100	64	0.06	0.27	0.10
X		206 r	191	100	26	0.12	0.61	0.15
X		309 r	286	100	12	0.20	1.18	0.41
X		360 r	333	84	7	0.12	1.38	0.524
β	21 rep/min	110 rep	100	100	77	0.07	0.15	0.04
β		220 rep	200	100	46	0.16	0.44	0.16
β		330 rep	300	50	18	0.20	0.68	0.22
β		385 rep	350	100	13	0.18	0.98	0.41
γ	20 r/min	300 r	300	100	33	0.09	0.70	0.14

Tradescantia inflorescences. Four completely independent experiments were performed to determine the relative effects of mixed 1.17 and 1.33 Mev γ-rays and X-rays of two different mean energies (0.2 A or 200 kev and 0.06 A or 60 kev respectively). A definite dependence of chromosomal breakage on wave length was also found for this material. Assuming γ-rays to have a relative effect of unity, the ratio of biological effectiveness of 0.20 A X-rays to 0.06 A X-rays to γ-rays is approximately 2 : 1.5 : 1.0.

It should be noted that comparisons are made in all cases between doses expressed in energy units. The measured aberration frequencies at the 0.2 A X-ray dose points have been corrected by the factors 300 : 278, 305 : 282 and 150 : 139 respectively, in order that equal tissue doses of the different radiations might be more accurately compared. Table 2 contains the data from these four microspore experiments.

TABLE 2

Comparative effects of 0.06 A X-rays, 0.20 A X-rays and cobalt^{60} gamma rays on Tradescantia microspores.

(Results are for four-day chromosomes.)

Radiation	Dose rate	Measured dose in air	Dose delivered to tissue in units of 93 ergs/gm	Cells		Aberrations per cell			
				Scored	Normal	Deletions	(Rel. eff.)	Exchanges	(Rel. eff.)
			Experiment 1						
0.06 A X	10 r/min	300	300	300	122	0.507	1.62	0.363	1.20
γ	10 r/min	300	300	300	159	0.313	1.0	0.303	1.0
			Experiment 2						
0.20 A X	31 r/min	300	278	240	69	0.679	1.95	0.458	1.92
0.06 A X	31 r/min	300	300	125	50	0.624	1.79	0.344	1.43
γ	29 r/min	300	300	300	155	0.373	1.0	0.257	1.0
			Experiment 3						
0.20 A X	11 r/min	305	282	340	106	0.553	1.97	0.482	2.06
0.06 A X	11 r/min	300	300	370	158	0.419	1.50	0.365	1.56
γ	10 r/min	300	300	302	172	0.30	1.0	0.25	1.0
			Experiment 4						
0.20 A X	31 r/min	150	139	300	190	0.277	1.44	0.18	2.47
0.06 A X	31 r/min	150	150	240	176	0.213	1.10	0.113	1.53
γ	29 r/min	150	150	252	186	0.206	1.0	0.079	1.0

DISCUSSION

Dependence of chromosomal breakage on energy of incident radiation

As has been noted, a definite dependence of chromosomal breakage on wave length or, more precisely, on energy of the incident radiation has been observed. Difference between the effects of X-rays of the different wave lengths and Co^{60} γ-rays have been found consistently in all the experiments performed. In view of the care taken in the dosage measurements, we are faced with the necessity for some revision in our ideas of the variation of chromosomal breakage with incident energy, and must certainly re-examine the evidence supporting the widely held belief that, at wave lengths shorter than 1.5 A, there is little

or no dependence of these biological effects on the frequency of the incident radiation.

The biological effects of ionizing radiation depends to a great extent upon the distribution of ionization produced in the tissue under study. A well-known curve showing the theoretical wave-length dependence of chromosomal breakage in the 8 A to 1.5 A region has been derived by LEA and CATCHESIDE (1942) from a consideration of the calculated ionization distribution in tissue as a function of the wave length of the primary radiation. This curve shows a steady diminution in chromosomal aberration production as the wave length of the incident radiation is decreased below 4 A. The general shape of the curve in the X-ray region has been studied experimentally in the case of Tradescantia pollen-tube chromosomes by CATCHESIDE and LEA (1943), who found chromosomal breakage produced by X-rays of 0.15 A to be only slightly less than that produced by radiations of 1.5 A wave length. Similar results for Tradescantia microspores were obtained by KOTVAL (unpublished work cited by LEA 1947) in experiments comparing the effects of 0.15 A X-rays and 0.015 A γ-rays from radium. These deviations from the predictions of the simple theory have been qualitatively explained by the production of secondary electrons (δ-rays) and by the effects of the less densely ionizing portions of the electron tracks. At the present time it is not possible to determine theoretically the exact value of the wave length at which such a leveling off of aberration production should occur. FANO (1943) has shown that this situation should be attained ultimately at some value of the energy of the incident radiation beyond which the ratio of ionization in clusters to that distributed along the electron tracks should remain a constant as the wave length is decreased. From this evidence it has been generally considered for some time that chromosomal breakage in Tradescantia and many other materials is essentially independent of the wave length of the exciting radiation in the region shorter than 1.5 A. A closer inspection of the experimental evidence shows this conclusion to be less reliable than it is often assumed. The pollen-tube work of CATCHESIDE and LEA (1943) cited above is subject to some criticism due to the small number of cells studied. The recent demonstration of large variations in pollen sensitivity from day to day further complicates the interpretation of early pollen-tube work.

The effect of X-rays of effective wave lengths 0.44 and 0.07 A, respectively, have been studied by FABERGÉ (1940), who found no significant differences in the numbers of chromosomal fragments produced in Tradescantia microspores. FABERGÉ applied doses of 1500 r in these experiments and analyzed for "number of fragments" only instead of scoring specific types of aberrations as was the case in our present work. Unless some saturation effects may have been brought about by the high doses, the disagreement between FABERGÉ's results and those results shown in table 2 cannot be explained at the present time. LEA and CATCHESIDE (1943) have pointed out that both points chosen by FABERGÉ are in a wave-length region in which the mean energy of electrons produced in tissue changes only slightly, and hence little or no effect should be observed.

The present work is not so greatly subject to this same objection, since the mean electron energies for our X-ray points, taken from LEA's (1947) tables, are 11 and 45 kv, respectively, compared with 15 and 29 kv in the work of FABERGÉ.

There are previous results supporting our present measurements. CATCHESIDE, LEA and THODAY (1946a), in some usually ignored results, have shown for Tradescantia microspores that the ratio of biological effectiveness of 200 kv X-rays (mean wave length 0.15 A) to radium γ-rays is 1.0 : 0.77. This is not greatly different from the ratio of 1.0 : 0.70 found in our work for 0.060 A X-rays compared with γ-rays. In another plant material there is some confirmation in the work of GRAY, REED and POYNTER (1943) on the lethal action of X- and γ-rays on the broad-bean root. Here the relative efficiency of 0.16 A X-rays to radium γ-rays is in the ratio of 1.5 : 1.0. This is not too different from our results of 2 : 1 for 0.20 A X-rays compared to cobalt γ-rays.

Little more than a tabulation of the wave-length aberration rate data can be made until a quantitative theory of the effects of ionization density in the high energy electron range, i.e., in the short wave-length range, has been constructed, or until more precise knowledge of the energy distribution in the X-ray beams is available. Even with filtration, the X-ray output from either constant potential or self-rectified machines is a wide smear of many different wave lengths. Some hope for accurate measurement of these X-ray energy distributions is now possible with the development of scintillation spectrometers. A second approach to a precise determination of wave-length effects consists in the use of monochromatic γ-rays from radioactive sources at a number of energies in the 1-Mev to 250-kv region. Consideration of both these methods is now underway.

Biological equivalence of P^{32} beta rays and Co^{60} gamma rays on Tradescantia pollen

The equivalence of these radiations on the production of chromosomal breakage is clearly shown in table 1 and figure 4. A simple qualitative explanation of this fact may be found in an examination of the electron energy distribution in these different sources. The γ-ray spectrum from Co^{60} consists ideally in two lines of 1.17 and 1.33 Mev energy, respectively. This situation is not realized in the practical case, due to the presence of some degraded scattered γ-rays as well as secondary electrons from the Compton process. In the present argument the exact value of the mean γ-ray energy is not critical and, for simplicity, we will assume a mean energy of 1 Mev. Using the tables computed by LEA (1947), the mean energy of electrons produced in tissues by 1 Mev γ-rays is found to be approximately 450 kv. These electrons are the biologically effective agents in the present experiments, and if the distribution in energy of the P^{32} electrons can be obtained, a valid comparison of the expected effects may be made.

The initial distribution of P^{32} β-rays from a thin source is well known, but these data cannot be applied directly to the thick P^{32} plaques used in the pres-

ent work. In the absence of a well-established theory of the degraded electron energy distribution in a thick source, the needed information must be found from experiment. A direct determination of the energy distribution from the P^{32} plaques, using a scintillation spectrometer, has been made recently by DARDEN (unpublished observations) in this laboratory and a mean electron energy of approximately 400 kv was found. Some additional confirmation of this result is found in a recent paper by BROWNELL (1952) on the energy distribution of β-rays from a thin P^{32} source after traversal through appreciable thicknesses of plastic.

It is thus apparent that little difference in ionization density, and consequently in biological effect, should result in a comparison of the effects of 450 kv electron from the Co^{60} γ-rays and the 400 kv P^{32} electrons. It should be noted however that these considerations are strictly valid only for thin tissues in which further degradation in energy of the β-rays is slight. This condition is satisfied in the case of pollen.

Isochromatid dose-breakage relations

CATCHESIDE, LEA and THODAY (1946b) have shown, in the case of Tradescantia microspores, a slight although significant departure from linearity in the isochromatid dose-aberration frequency relation. As can be seen in all the curves representing the present data, this effect is definitely confirmed in pollen-tube experiments. A least-squares analysis of the data for 0.20 A X-rays (exp. A, B, C and E) shows that a better fit is achieved by the relation $y = aX + bX^2$, where y is the aberration yield per cell and X is the dose in energy units, than by a strictly linear relation.

These data are summarized in table 3, along with the figures for β- and γ-ray isochromatid dose curves. The values of a and b derived by CATCHESIDE *et al.* (1946b) for microspores are also included for comparison. It is interesting to note that, although the absolute values differ, the ratios a/b for the present pollen results and the ratio a/b for microspores are almost identical.

TABLE 3

Coefficients of X and X^2 in the relation $y = aX + bX^2$, where y is the isochromatid aberration yield per cell and X is the dose in energy units.

Radiation	Expt.	Coefficient of $X \times 10^{-2}$	Coefficient of $X^2 \times 10^{-4}$	Test of significance	
				Coeff. of X	Coeff. of X^2
X	A	.293 ± .085	.082 ± .027	.02 – .05	.05 – .10
X	B	.393 ± .082	.036 ± .029	< .01	.20 – .30
X	C	.339 ± .088	.086 ± .032	.01 – .02	.05 – .10
X	E	.215 ± .046	.063 ± .016	.02 – .05	.05 – .10
β	A	–.026 ± .097	.111 ± .030	> .50	.05 – .10
β	D	.072 ± .029	.015 ± .009	.10 – .20	.20 – .30
β	E	.1107± .052	.045 ± .017	.10 – .20	.10 – .20
γ	A	.028 ± .023	.069 ± .007	.30 – .40	< .01
γ	D	.011 ± .050	.045 ± .016	> .50	.10 – .20
Microspores (Catcheside)		.177	.048		

A curious trend in the data shown in figure 3 is the apparently greater importance of the X^2 term (two-hit component) in the expression $y = aX + bx^2$ for β- and γ-ray-induced isochromatid aberrations as contrasted with that for X-rays. Further experimental work of a very tedious nature would be required to settle this point definitely, but from the present results some difference in the mechanism of chromosomal breakage between 0.20 A X-rays and γ-rays or high energy beta rays is indicated. Recent calculations of the ionization density produced by X-, γ- and β-rays by CORMACK and JOHNS (1952), as well as earlier work by GRAY (1947), support such an hypothesis. These investigators have shown that the mean ionization density (ions per micron of electron track) for 200 kvp X-rays is 100 compared with a figure of 11 for cobalt γ-rays. It would be surprising if this approximately tenfold decrease in ionization density did not result in some very definite increase in the two-hit component.

SUMMARY

The effects of mixed 1.17 and 1.33 Mev γ-rays from Co^{60}, medium energy X-rays (60–250 kev) and P^{32} β-rays (mean energy 400 kev) on chromosomal breakage in Tradescantia have been compared. A number of independent irradiations of Tradescantia pollen using these agents have led to the following results and conclusions:

X-rays of 60 kev mean energy are twice as effective as Co^{60} γ-rays or 400 kev mean energy β-rays in producing chromosomal aberrations. 1 Mev γ-rays and 400 kev mean energy β-rays are closely identical in their biological effects on Tradescantia dry pollen. This finding is in agreement with predictions based upon considerations of relative ion density produced by these radiations.

A definite nonlinear component in the dose-aberration frequency relation for isochromatid breaks has been observed for the three different agents. This result indicates a definite two-hit component in the aberrations produced.

An analysis of the isochromatid dose-aberration frequency curves for the three radiations shows the relative magnitude of the two-hit component to be considerably greater for γ-rays or high-energy β-rays than for medium energy X-rays. Consideration of relative ion densities supports this result.

The dependence of chromosomal breakage on wave length of incident radiation in Tradescantia dry pollen has been confirmed in microspore studies.

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CYTOGENETICS AND PHYLOGENY OF OENOTHERA ARGILLICOLA MACKENZ.[1]

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THE evening primroses, long known for their cytogenetic peculiarities, have in recent years been the subject of extensive investigations relating to the phylogeny of the North American members of the subgenus Euoenothera (see review by CLELAND 1949). It has been found that the anomalous cytogenetic behavior characteristic of most members of the group can serve as a useful tool in analyzing population structure and in determining relationships which exist among various euoenotheras (CLELAND 1936). The present paper reports results of a study of certain members of the species *Oe. argillicola,* occurring as a shale barren endemic in the Appalachian Mountains of Virginia, West Virginia, Maryland and southern Pennsylvania.

With respect to cytogenetic behavior the Euoenothera population may be divided into two major categories: (1) the forms residing in California and northern Mexico, which possess all-pairing, lethal-free chromosomes, and large, open-pollinated flowers; (2) the forms occupying the region east of the Rocky Mountains, which possess circles of fourteen chromosomes at meiosis, balanced lethal systems, and a self-pollinating habit.

The second situation does not, however, prevail over the entire eastern part of the continent; there are exceptional groups that fail to show the aberrations mentioned. One such group, found in the southeastern United States, has been reported by STEINER (1952). The *argillicola* assemblage here under discussion constitutes a second exceptional eastern group.

Previous studies on Euoenothera phylogeny have shown that the North American population may be divided into several phylogenetic units, some of these units almost certainly deserving of species status (see CLELAND *et al.* 1950, pp. 218–254). The purely taxonomic phases of the Oenothera studies, under the direction of P. A. MUNZ, are as yet incomplete; therefore, this paper will follow the practice of naming the various phylogenetic groups according to the nomenclature adopted in the earlier investigations. Several of these groupings are pertinent to the present investigation, and require some discussion.

Hookeris—These plants are found in California and northern Mexico. They do not display the cytogenetic aberrations usually associated with Oenothera,

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for they form seven bivalents at meiosis, lack balanced lethals, and possess large, open-pollinated flowers. These cytogenetic characters are undoubtedly primitive, and their presence in the hookeris suggests that these forms closely approach the original ancestral condition for North American euoenotheras. The area occupied by this group is assumed to be not far removed from the center of distribution for the subgenus in North America. The most frequent segmental arrangement found in the group (the ^{h}*Johansen*[2] arrangement)—1·2 3·4 5·6 7·10 9·8 11·12 13·14—is taken to be the hypothetical ancestral arrangement. That the latter assumption is valid is suggested by the fact that the chromosomes of the ^{h}*Johansen* arrangement are the most frequently found of all chromosomes throughout the range of the euoenotheras (CLELAND *et al.* 1950). Apparently these chromosomes are relics which have escaped interchange.

Biennis groups—Three divisions have been recognized within the *biennis* grouping, primarily on the basis of cytogenetic specializations. Races in group 1 are mid-western, ranging from the Ozark region eastward to the Appalachian Mountains, and breaking through the mountains to the Atlantic Coast in Virginia. They show broad, thin, crinkly leaves and rather sparse, erect pubescence. The distinctive *biennis* features are produced by the egg (alpha) complexes, which effectively mask the *strigosa* phenotypic characters of the pollen (beta) complexes. The alpha complexes in this group are closely related to the ancestral arrangement, whereas the betas are more closely related to the beta strigosas.

Races in *biennis* group 2 are for all practical purposes phenotypically indistinguishable from members of group 1. They differ from group 1, however, in that they possess alpha complexes yielding *strigosa* phenotypes, and beta complexes yielding *biennis* phenotypes—just the reverse of the situation in group 1. *Biennis* group 2 is northeastern in distribution, ranging from northern Wisconsin and Michigan to New Brunswick and south along the Atlantic Coastal Plain as far as North Carolina. Segmentally, the alpha complexes are fairly closely related to the beta strigosas. The betas probably represent an earlier indigenous population which crossed with invading strigosas.

The forms in *biennis* group 3, which occur in Virginia and North Carolina, show a more accentuated *biennis* appearance, since both complexes in these races produce *biennis* phenotypes. In segmental arrangement the alpha complexes are close to the alpha group 1 complexes, and the betas are essentially identical with the beta group 2 complexes. It is very probable that group 3 arose as a result of crossing between groups 1 and 2, or their progenitors, in the region of overlap in their ranges, a *biennis* 1 alpha complex becoming associated with a group 2 beta complex.

*Parviflora*s—This group occupies the same general range as *biennis* group 2, but is quite distinct phenotypically and segmentally from the latter. The parvi-

[2] The superscript "h" (haplo) denotes that the complex is present in a structurally homozygous form.

floras are characterized by narrow, somewhat leathery leaves, sparse, appressed pubescence, bent stem tips and subterminal sepal tips—characters which are all due to the beta complexes. The alpha complexes are of two types, one producing a *strigosa*-like phenotype and the other a *biennis*-like appearance. Segmentally, the beta complexes differ greatly among themselves and show little resemblance to any other known group. The alphas, which show a somewhat closer affinity to the original, are also heterogeneous, but there is one arrangement which appears rather widespread. Recent studies by the writer indicate that the parvifloras which reside as far south as Virginia differ in their phylogenetic relationships from the races located in the Northeast.

MATERIAL AND METHODS

The sources of the plants used in this study are shown in table 1. Two strains, *Huntingdon* A and B, have been analyzed earlier by CLELAND, and the

TABLE 1

Sources of races completely analyzed.

Race	Locality	Collector	Year	Configuration
Douthat 1	Shale bank along Wilson Creek, Allegheny Co., Va.	R. B. Platt	1947	7 prs.
Douthat 2	Shale bank along Cow Pasture River, Allegheny Co., Va.	R. B. Platt	1947	7 prs.
Douthat 3	Same as Douthat 2	R. B. Platt	1947	7 prs.
Douthat 4	Same as Douthat 2	R. B. Platt	1948	⊙8, 3 prs.
Huntingdon A *	Near city of Huntingdon, Huntingdon Co., Penna.	E. T. Wherry	1933	7 prs.
Huntingdon B	Same as Huntingdon A	E. T. Wherry	1933	7 prs.
White Sulphur Springs **	Near White Sulphur Springs, Greenbrier Co., W. Va.	H. H. Bartlett	1928	⊙4, 5 prs.

* *Huntingdon* A = *argillicola* A of previous papers of CLELAND and GECKLER.
** See MICKAN 1935.

strain from White Sulphur Springs, West Virginia was studied by MICKAN in Germany (MICKAN 1935). In the case of MICKAN's race it was found that the system of end arrangements used by the German workers differed from the system adopted in the initial investigations of CLELAND and BLAKESLEE; hence, it was necessary to derive end arrangement formulae based on our system from the hybrid configurations reported by MICKAN. It should be mentioned that it was impossible to derive a formula for the complex *dilatans* from the data reported by MICKAN. There is one formula, however, which will satisfy all configurations reported by MICKAN except one. If, therefore, this single configuration is assumed to have been incorrectly stated it is possible to arrive at a formula for *dilatans*. Thus the segmental arrangement for *dilatans* presented in this paper must be considered provisional, since it cannot be certain that the data upon which the formula is based are entirely correct.

In addition to these materials, other races collected by the writer throughout the range of the species were subjected to a preliminary cytological study. These races, although incompletely analyzed, are included since they throw some light on the population structure with respect to chromosome configurations and ecological preferences.

For the analysis of phenotypic effects and segmental end arrangements, each new race was crossed as male and as female parent with ten to twenty " standard " races whose complexes had previously been determined as to phenotype and segmental arrangement. All material for cytological study was obtained from smears of young anthers which were fixed and stained according to the schedule given by HECHT (1950). Chromosome configurations were determined from microsporocytes in late diakinesis or metaphase I.

The detailed reasoning upon which the segmental end arrangements are based, as well as complete descriptions of the races, are on file in the Botany Department of Indiana University.

OBSERVATIONS AND DISCUSSION

Geographical distribution and habitat preference

Oe. argillicola was first described by MACKENZIE (1904) from a collection near White Sulphur Springs, West Virginia. The range of this species extends approximately from the vicinity of Roanoke in southwest Virginia to the vicinity of Huntingdon in southcentral Pennsylvania (WHERRY 1930, 1933). Geographically it overlaps the ranges of several Euoenothera groups, including the three *biennis* groups, the parvifloras, and the *biennis*-like races in the Southeast. Ecological preferences, however, tend to separate some of these species in spite of geographical propinquity. The preferences relate to the ability of the several races to inhabit shale barren areas, the three major groups of euoenotheras resident in this area showing different reactions to the shale barren habitat. The *argillicola* group is restricted closely to the shale banks and is not found to any extent on other soils. On the other hand, plants of the *biennis* groups appear to invade the shale barrens very rarely, although they are often seen growing only a few hundred feet distant from the shale banks. The third group of races, the parvifloras, behaves differently from either the argillicolas or the *biennis* groups. The southern members of the *parviflora* population are able to exist with the argillicolas on the shale barrens. However, unlike the argillicolas, they are not strict endemics of the barrens, and frequently occur in the weedy habitats with the *biennis* forms. It appears that the parvifloras, at least those occurring in the southern extension of the range, are well adapted to both environments, whereas the *argillicola* and *biennis* races thrive in one or the other, but not both. The fact that certain *parviflora* races are able to occupy the rather special habitat of the argillicolas is strongly indicative of relationship between the two groups.

In an ecological study of the shale barren flora PLATT (1951) has analyzed various factors which may contribute to the endemism of these plants. He finds

that the endemics are " obligate upon a high light intensity, a soil adequate for their extensive root systems and a low level of competition." The sparsity of plant life on the shale barrens is explained as due primarily to restriction of seedling development imposed by the low moisture conditions and high temperatures which obtain in the surface layer.

Cytogenetic behavior

The *argillicola* races are among the few euoenotheras east of the Rockies which fail to show circles of fourteen chromosomes at meiosis. Among the argillicolas so far investigated meiotic configurations ranging from seven bivalents to a circle of ten and two pairs have been observed, with the majority of the races showing either seven pairs or small circles and several pairs. Some impression of the overall frequency of occurrence of the various configurations in natural populations is gained from the data in table 2. It will be seen that the two most frequent configurations are seven pairs, and a circle of four and five pairs, an indication that the complexes in the population are, on the whole,

TABLE 2

Summary of argillicola configurations.

Configuration	Number of plants of 1st generation in which encountered
7 prs.	29
⊙4, 5 prs.	27
⊙4, ⊙4, 3 prs.	12
⊙4, ⊙4, ⊙4, 1 pr.	1
⊙6, 4 prs.	15
⊙6, ⊙4, 2 prs.	9
⊙8, 3 prs.	4
⊙10, 2 prs.	1

very similar in segmental end arrangement. The next most frequent configurations, a circle of six and four pairs, and two circles of four and three pairs, also indicate close relationship between the complexes involved, for the complexes giving each configuration are a minimum of two interchanges removed from each other.

Accompanying this tendency to produce paired chromosomes is the almost complete absence of balanced lethals. Only the strain from West Virginia analyzed by MICKAN has been found to possess balanced lethals. This race showed a circle of four and five pairs at meiosis, the smallest ring structure possible in Oenothera. This is the only reported case of balanced lethals in a circle of this size. MICKAN's evidence clearly indicates that the lethal system in his race operates on the zygotic level, since each complex is transmitted by both the egg and sperm.

No evidence of balanced lethals in circle-bearing forms has been obtained from our material. But a thorough survey specifically designed to detect lethals, both in forms with seven pairs and with circles, is needed in view of RENNER's

suggestion (1933, p. 248) that the lethal-bearing *argillicola* race of MICKAN offers evidence that lethal action appears to go hand in hand with the translocation process.

A third important feature of the breeding behavior of the argillicolas is the fact that they are open-pollinated. This again is unusual for oenotheras in the eastern part of the continent.

The *argillicola* population is thus seen to consist of ecologically restricted, essentially lethal-free, open-pollinated individuals with all-pairing chromosomes or small circles and several pairs. In natural populations, open-pollination should lead to considerable crossing among individuals occupying the same area. At the same time, as a result of the absence of lethals, one would expect that whenever self-pollination in the circle-bearing forms did take place segregation for chromosome configuration would occur.

That both situations mentioned, hybridization among biotypes and segregation for chromosome configuration, do in fact take place in wild populations is indicated by cytological analysis of material grown from seed collected throughout the range of the species. The results of this analysis are shown in table 3, where each culture represents plants grown from seed collected from a single plant. In the cultures listed in parts A, B and C of this table more than one configuration was observed; part D shows cultures in which only one configuration was found.

The cultures in part A in all probability represent cases of segregation for chromosome configuration; for example, in culture 96, the 7-paired form could well be a segregate from the configuration of ⊙8, 3 pairs, provided the circle, or at least half of it, was lethal free. The configurations shown in part B may also represent segregation for chromosome configuration, but here one cannot be sure, since the parental configurations were not observed in all instances. In number 815, for example, it is possible that the two configurations ⊙6, 4 pairs and ⊙4, 5 pairs are the result of segregation from a parent with either ⊙6, ⊙4, 2 pairs or ⊙6, ⊙4, ⊙4, neither of these parental configurations appearing in the plants examined cytologically. Without the configuration of the seed plant, it is not possible to state whether this was actually the situation or whether the configurations resulted from cross-pollination.

In cultures 783 and 835 (part C) segregation of chromosome configuration is almost certainly not the answer. Thus in 835, although the configurations ⊙6, 4 pairs and ⊙4, ⊙4, 3 pairs could have arisen from a parental plant with ⊙6, ⊙4, ⊙4, the plant showing ⊙8, 3 pairs could not have similarly arisen. Likewise, the configurations ⊙6, 4 pairs and ⊙8, 3 pairs could have arisen from a parental plant with ⊙8, ⊙6, but the plant with ⊙4, ⊙4, 3 pairs could not have been so derived. In other words, there is no single configuration from which the three configurations actually observed in culture 835 could have arisen as a result of segregation alone. The same is true for the configurations reported for culture 783; it is not possible to get ⊙4, 5 pairs, ⊙6, 4 pairs and ⊙8, 3 pairs from any one parental configuration by means of segregation. The most likely explanation to account for these observations is that certain of the

TABLE 3

Chromosome configurations in argillicola populations.

	Culture number	No. plants in culture	No. plants determined	Configurations
	96	15	2	⊙8, 3 prs. (1)*; 7 prs. (1)
	97	15	2	⊙6, 4 prs. (1); 7 prs. (1)
	776	15	5	⊙6, ⊙4, 2 prs. (4); ⊙4, 5 prs. (1)
	777	15	5	⊙6, 4 prs. (3); ⊙4, 5 prs. (1) ⊙6, ⊙4, 2 prs. (1)
	780	15	6	⊙4, 5 prs. (5); 7 prs. (1)
	782	15	6	⊙4, 5 prs. (3); 7 prs. (3)
	787	15	2	⊙6, 4 prs. (1); 7 prs. (1)
A	836	15	2	⊙4, ⊙4, 3 prs. (1); ⊙4, 5 prs. (1)
	843	15	2	⊙4, 5 prs. (1); 7 prs. (1)
	845	15	2	⊙4, ⊙4, ⊙4, 1 pr. (1); ⊙4, ⊙4, 3 prs. (1)
	849	15	2	⊙6, ⊙4, 2 prs. (1); ⊙6, 4 prs. (1)
	850	15	2	⊙4, ⊙4, 3 prs. (1); 7 prs. (1)
	852	15	2	⊙4, ⊙4, 3 prs. (1); ⊙4, 5 prs. (1)
	856	15	2	⊙4, 5 prs. (1); 7 prs. (1)
	858	15	2	⊙4, 5 prs. (1); 7 prs. (1)
	859	15	4	⊙4, ⊙4, 3 prs. (3); ⊙4, 5 prs. (1)
	865	13	3	⊙4, 5 prs. (1); 7 prs. (2)
	866	15	2	⊙8, 3 prs. (1); 7 prs. (1)
	781	14	4	⊙4, 5 prs. (2); ⊙10, 2 prs. (1); 7 prs. (1)
	812	15	5	⊙4, ⊙4, 3 prs. (2); ⊙6, ⊙4, 2 prs. (1);
B				⊙4, 5 prs. (1); 7 prs. (1)
	815	15	6	⊙6, 4 prs. (5); ⊙4, 5 prs. (1)
	783	10	4	⊙4, 5 prs. (1); ⊙6, 4 prs. (1); ⊙8, 3 prs. (1); 7 prs. (1)
C	835	15	4	⊙6, 4 prs. (2); ⊙4, ⊙4, 3 prs. (1); ⊙8, 3 prs. (1)
	80	15	2	7 prs. (2)
	95	14	1	7 prs. (1)
	755	1	1	7 prs. (1)
	769	15	1	7 prs. (1)
	771	15	2	⊙6, ⊙4, 2 prs. (2)
	779	16	2	7 prs. (2)
	786	15	1	7 prs. (1)
	788	15	1	7 prs. (1)
D	813	15	1	⊙6, 4 prs. (1)
	814	15	2	⊙4, 5 prs. (2)
	819	15	2	7 prs. (2)
	839	20	2	⊙4, 5 prs. (2)
	842	15	1	⊙4, ⊙4, 3 prs. (1)
	848	15	1	⊙4, ⊙4, 3 prs. (1)
	860	20	1	7 prs. (1)
	871	20	1	⊙4, 5 prs. (1)

*The numbers in parentheses after each configuration give the number of plants showing the configuration.

configurations are the result of hybridization between the plant from which seeds were collected and some other member of the population, complexes of different end arrangements thus being brought together.

It is, of course, possible that any particular configuration which appears to

be a segregate from some other configuration could have been produced through hybridization as well. In other words, when dealing with plants collected in the wild, the fact that one chromosome configuration within a single line could be derived from another configuration in the same line by self-pollination is not absolute proof that this has actually been the course of events.

These observations are in striking contrast to the usual situation in Oenothera where one commonly is working with self-pollinating complex-heterozygotes, which because of circles of fourteen and balanced lethals, breed true to their chromosome configurations generation after generation. Even races first established in the experimental garden are true breeding, for the self-pollinating habit appears to have presented a formidable barrier to crossing between biotypes in the population where the race was collected. On the other hand, in some populations composed of open-pollinated individuals with all-pairing, lethal-free chromosomes, such as the hookeris, the population may be so homogeneous in regard to segmental end arrangements that hybrid plants will still reveal seven pairs at meiosis. The detection of outcrossing in the *argillicola* population is possible because of the existence throughout the population of complexes with different end arrangements.

The three cytogenetic features discussed—the presence of all or mostly paired chromosomes, open-pollination, and the almost complete absence of balanced lethals—although uncommon in most euoenotheras, are, nevertheless, characteristic of the hookeris of northern Mexico and California. The hookeris, it will be recalled, are considered to be primitive in respect to these characters. Therefore, in displaying these primitive traits of cytological and genetical behavior, the argillicolas show affinities to the putative ancestral population. They have not, in other words, developed the aberrations so prominent in the majority of euoenotheras.

Segmental end arrangements

The segmental end arrangements thus far worked out for the argillicolas are shown in table 4. The arrangements are seen to be fairly similar to one another, a fact which is substantiated by the cytological survey of the entire population described above. Three complexes in the table have identical end arrangements, and the remaining six are either one or two interchanges removed from this arrangement. The fact that the arrangements of the southernmost members of the group, the *Douthat* races, are close to those of the northernmost races, the *Huntingdon* strains, suggests that the arrangements worked out are fairly typical of the group as a whole.

A second important point is the close similarity between the *argillicola* arrangements and those found in the hookeris. One race, *Douthat 3,* has the ^{h}*Johansen* arrangement, which is taken to represent the hypothetical ancestral arrangement for the North American euoenotheras. The end arrangements of *Douthat* 1, *Douthat* 2, *dilatans, Douthat* 4a, and *Huntingdon* B, are all one interchange removed from the ^{h}*Johansen* arrangement, while *angustans, Huntingdon* A and *Douthat* 4b are two interchanges removed. Thus from the point

TABLE 4

Argillicola segmental end arrangements.

hDouthat 3	1 • 2	3 • 4	5 • 6	7 • 10	9 • 8	11 • 12	13 • 14
hDouthat 1	1 • 4	3 • 2	"	"	"	"	"
hDouthat 2	"	"	"	"	"	"	"
dilatans	"	"	"	"	"	"	"
angustans	"	"	"	7 • 8	9 • 10	"	"
hHuntingdon A	"	3 • 6	5 • 2	7 • 10	9 • 8	"	"
hHuntingdon B	1 • 8	3 • 4	5 • 6	"	9 • 2	"	"
hDouthat 4a *	1 • 13	"	"	"	9 • 8	"	2 • 14
hDouthat 4b	1 • 4	3 • 12	"	"	"	11 • 2	13 • 14

* The strains *Douthat* 4a and *Douthat* 4b are seven paired segregates from *Douthat* 4 (see table 1) which were isolated in the experimental garden.

of view of segmental arrangements as well as in the presence of paired chromosomes and open-pollination, and the rarity of lethals, the argillicolas show a very close relationship with the western hookeris, i.e., they are primitive in so far as these characters are concerned.

Phenotypic characters

Although the argillicolas are closely related to the *hookeri* group in cytogentic behavior and reproduction, the same cannot be said in regard to vegetative morphology, in which they depart widely from the *hookeri* condition. They are easily distinguished from other euoenotheras by the following characters: long, narrow, somewhat leathery, essentially glabrous foliage; delicate, brittle, glabrous stems; large, glabrous buds with long, subterminal sepal tips; bent stem tips; semi-procumbent growth habit; and capsules which diverge widely from the stem and gradually curve upward. Some of these characters, notably bent stem tips and subterminal sepal tips, are unusual in this subgenus. However, the *argillicola* complexes are not unique in phenotypic expression, for their effect on foliage, stem and sepal tip characters is similar to that produced by the beta *parviflora* complexes. These distinctive characters are much more pronounced in the *argillicola* races than in the *parviflora* races, since the argillicolas are very nearly homozygous, whereas in the parvifloras the beta complexes are associated with alpha complexes which give rise to entirely different phenotypes, producing characters which to some extent mask those of the beta complexes. The argillicolas and beta parvifloras differ in phenotypic expression mainly in that the former possess large buds and flowers, and the latter, extremely small buds and flowers.

Morphological evidence, therefore, relates the argillicolas with the beta *parviflora* complexes rather than with the hookeris.

Chlorophyll inheritance

(a) *Comparison of effects of argillicola and beta parviflora genomes on plastids.* Still another resemblance between the *argillicola* and beta *parviflora* complexes is found in their behavior with respect to the production of chlorophyll deficiencies. This follows from the observation that plastids of certain

groups of races fail to synthesize normal amounts of chlorophyll when present with complex-combinations involving either beta *parviflora* or *argillicola* genomes. The combined data of GECKLER (1950), CLELAND (personal communication), and the writer indicate that, in general, beta *parviflora* and *argillicola* genomes have a role in causing chlorophyll deficiencies in plastids of the following races: *biennis* group 1, *strigosa* and *hookeri* strains; *blandina* (a *lamarckiana* mutant); *Camas* (an unclassified form); *grandiflora* of DE VRIES; *biennis* group 2 forms, primarily when the broad-leaved (beta) complexes of the latter are present in the complex-combination. On the other hand, both the beta *parviflora* and *argillicola* genomes, as a rule, exert no deleterious effects on the plastids of *biennis* group 2 races when the narrow-leaved (alpha) complex of the latter is present. Finally, beta *parviflora* genomes allow normal development of *argillicola* plastids and vice versa, as might be expected.

The facts just mentioned represent generalizations based on comparison of the results of similar crosses involving the argillicolas and parvifloras as male parents. These observations show rather clearly that the *argillicola* and beta *parviflora* genomes possess genes which in combination with certain other genomes react with the plastids to produce disturbances in the elaboration of chlorophyll. The fact that the *argillicola* and beta *parviflora* complexes behave in a similar way, and in a way different from all other known complexes, suggests a phylogenetic connection between the two.

Certain points of genetic interest also emerge from the chlorophyll studies. The results with *biennis* group 2 show that while *argillicola* and beta *parviflora* genomes influence the induction of chlorophyll deficiencies, the complex contributed by the female parent also plays a role. For instance, plastids of the race *shulliana* (a *biennis* 2 race with the complexes *jugens* and *maculans*) are normal in the presence of a *jugens*·^{h}*argillicola* combination, but are nearly devoid of chlorophyll in the presence of a *maculans*·^{h}*argillicola* combination. Thus as RENNER (1924) has pointed out, plastid incompatibility is a function of the relationship between plastids and complex-combination, not between plastids and a single complex, even though certain complexes are more prone than others to produce complex-combinations which are deleterious to certain classes of plastids. It would be of interest to know whether or not these complex-combinations react in a similar way with all classes of plastids. One wonders, for example, if a *maculans*·^{h}*argillicola* combination would react with the cytoplasm of other groups in the same manner it does with the *biennis* group 2 race, *shulliana*.

(b) *Comparison of argillicola and parviflora chloroplasts.* Comparisons of similar crosses involving the argillicolas and parvifloras as female parents offer evidence that the plastids in races of the two groups react differently. *Parviflora* plastids are compatible with practically all genome combinations, even when these combinations include a beta *parviflora* complex. In contrast, *argillicola* plastids, on the whole, are deficient in chlorophyll when brought into association with the combination of an *argillicola* genome and one or the other genomes of races belonging to the *biennis* 1, *strigosa* or *hookeri* groups, as well

as the strains *Camas, grandiflora* of DE VRIES, *blandina* and *shulliana* (only when the broad-leaved complex of the latter is present). These races are the same ones whose own plastids are incompatible with *argillicola* genomes. In other words, reciprocal crosses between the argillicolas and these tester strains yield chlorophyll-deficient plants, while reciprocal crosses between these same strains and the parvifloras give chlorotic plants when the cross involves a *parviflora* as male parent and green plants when a *parviflora* serves as female parent.

These relationships may be illustrated by the series of crosses shown below, where the complex-homozygous race, *blandina,* is crossed reciprocally with a *parviflora* race and with a 7-paired *argillicola* form. (The *parviflora* race used here is *Clifton Forge;* the *argillicola* race is *Douthat* 3.)

Cross:	parviflora ♀ × blandina ♂	blandina ♀ × parviflora ♂
Plastids:	parviflora	blandina
Complex-combination:	beta *parviflora* · ^{h}*blandina*	^{h}*blandina* · beta *parviflora*
Description:	plants green	plants chlorotic
Cross:	argillicola ♀ × blandina ♂	blandina ♀ × argillicola ♂
Plastids:	argillicola	blandina
Complex-combination:	^{h}*argillicola* · ^{h}*blandina*	^{h}*blandina* · ^{h}*argillicola*
Description:	plants chlorotic	plants chlorotic

In the case of the *parviflora* race, it is evident that *blandina* plastids are incompatible with the ^{h}*blandina*·beta *parviflora* complex combination, whereas *parviflora* plastids are normal in association with this same combination. This must mean that substitution of seven beta *parviflora* chromosomes for seven *blandina* chromosomes in *blandina* cytoplasm interferes with chlorophyll production, while substitution of a *blandina* genome for the alpha *parviflora* genome in *parviflora* cytoplasm has no such deleterious effect.

With the *argillicola* race the situation is different. As previously explained, the *argillicola* genome has the same effect on *blandina* plastids as the beta *parviflora* complex (cf. crosses where *argillicola* and *parviflora* act as male parent with *blandina*). However, in contrast to the comparable *parviflora* cross, the substitution of seven *blandina* chromosomes for seven *argillicola* chromosomes prevents normal chlorophyll synthesis in *argillicola* plastids. Thus, not only are *blandina* plastids incompatible with *argillicola* genomes, when the latter are combined with ^{h}*blandina,* but also *argillicola* plastids are apparently incompatible with *blandina* genes in the same genome combination. As shown above, the latter condition does not hold for *parviflora* plastids. On the basis of the type of reaction illustrated with the *blandina* chromosomes, therefore, *argillicola* and *parviflora* plastids appear to fall into different classes.

The really critical test of this point would come from the results of crosses which placed the beta *parviflora*·^{h}*blandina* complex-combination in *argillicola* cytoplasm and the ^{h}*argillicola*·^{h}*blandina* combination in *parviflora* cytoplasm. For when this is done, one would expect *argillicola* plastids to be incompatible with beta *parviflora*·^{h}*blandina* genes, and *parviflora* plastids to be fully compatible with the ^{h}*argillicola*·^{h}*blandina* combination, if the results outlined above

do reflect differences in *argillicola* and *parviflora* plastids. A program designed to explore these and other possibilities relative to chlorophyll inheritance is projected for the near future.

Although these observations indicate differences between *argillicola* and *parviflora* plastids, it is probable that they are not too distantly separated in their evolution, since plastids and genomes in the two groups are mutually compatible. It is only when the plastids are considered in reference to non-*argillicola* and non-*parviflora* genomes that the differences become apparent.

ORIGIN OF THE ARGILLICOLAS

Possible origin from the biennis groups

In a discussion of Appalachian shale barren plants WHERRY (1930) lists possible sources of origin for shale barren endemics. *Oe. argillicola* is considered to have arisen from a "surrounding species," *Oe. biennis*. In our terminology this would mean one of the *biennis* groups 1, 2 or 3, all of which are "surrounding species" of the argillicolas. From the point of view of geographical location any one of the *biennis* groups could conceivably have been the source of the argillicolas. However, other lines of investigation afford little evidence that the *biennis* groups have served as progenitors of the *argillicola* races.

When the three *biennis* groups are considered from the standpoint of segmental arrangement, it is apparent that only the alpha complexes of group 1 and group 2 bear close resemblance to the argillicolas. The commonest *argillicola* end arrangement is identical with the alpha complexes of the *biennis* 2 races *Morgantown* and *St. Croix*, and is but two interchanges removed from the commonest alpha *biennis* 2 arrangement (that found in alpha *Waterbury*). The commonest arrangement among the alpha *biennis* 1 complexes is only one or two interchanges removed from most *argillicola* complexes. Thus, there is some evidence from similarity in segmental arrangements that the alpha complexes of *biennis* group 1 or group 2 could have given rise to the *argillicola* races.

Phenotypically, however, all complexes in the *biennis* groups are radically different from the argillicolas. The alphas of *biennis* 1 yield *biennis* characters, and the alphas of *biennis* 2 produce *strigosa* characters. In addition, both of these classes of alpha complexes produce self-pollinating flowers, whereas the argillicolas are open-pollinated.

Still further objections may be raised to the acceptance of either group of present day alpha *biennis* complexes as the progenitor of the argillicolas. The alpha complexes in question possess lethals, and exist in large circle-bearing individuals, whereas the majority of *argillicola* complexes are lethal-free and occur in homozygous forms. Therefore, if the argillicolas did originate from either *biennis* group 1 or 2, the *argillicola* races must have arisen as 7-paired segregates composed of alpha complexes in double dose from one of the *biennis* groups. This process would have to entail loss of lethals and the acquisition of

long styles and open-pollination. It is true that loss of lethals is known to take place on occasion in Oenothera, giving rise to 7-paired homozygotes. With regard to the acquisition of open-pollination it is possible that in nature a strong selection pressure would favor any homozygote which developed open-pollination, since this would allow introduction of genes from other individuals in the population and so maintain a higher level of heterosis. Hence, at first sight, it is theoretically possible for a 7-paired, open-pollinated population to arise from a circle-bearing, self-pollinated population.

Other factors, however, make it unlikely that a loss of lethals in either of the alpha *biennis* complexes would lead to the series of events just outlined. It may be asked in this connection what kinds of plants, from the point of view of genic content and breeding behavior, would result if an alpha complex of either *biennis* group 1 or 2 should lose its lethal, and thus come to exist in homozygous form. First of all, such plants would be absolutely homozygous (at least in the beginning), since they would have received in duplicate the same genome of seven chromosomes. As a consequence, any recessive genes in the complex would be present in double dose, and thus would be able to exert their effects. The chances are good that a complex which had previously existed in a race with ⊙14 would have accumulated various recessive mutations because of the enforced heterozygosity of the plant which contained them. These mutations would have enjoyed some degree of immunity from possible elimination by natural selection. It is reasonable to suppose, furthermore, that some of these recessives would be deleterious. So long as heterozygosity was enforced, these would not be able to reach their full expression. But once the complex, through loss of its lethals, came to exist in homozygous form these deleterious genes would no longer be covered, completely or partially, by genes in an opposing complex, and could then exert their full effects. This would mean that plants which contained the complex in homozygous form would from the very start be handicapped by deleterious genes, and many such plants would probably be weeded out by selection.

The accumulation of recessive genes, however, may not be as extensive as appears at first sight, in view of recent investigations in Drosophila which point to a measurable degree of dominance exhibited by recessive genes (STERN and NOVITSKI 1948; MULLER 1950; CORDEIRO 1952; PROUT 1952; STERN *et al.* 1952). These investigations have emphasized that the effects of deleterious recessive genes, as measured in evolutionary time, are more likely to be realized in the heterozygous than homozygous condition, since the average persistence of rare genes with only a slight degree of dominance is far less than the time required for the genes, on the average, to become homozygous through random mating. If these findings are generally applicable to genes in populations, then even with enforced heterozygosity deleterious recessive genes may still manifest their effects and thus bring about the elimination of some individuals carrying them. In fact, in the euoenotheras, where structural hybridity coupled with self-pollination virtually ensure against homozygosity, it might be expected that the partial dominance displayed by recessive genes would be

an effective check against the unlimited accumulation of deleterious recessive genes.

Such a mechanism, nevertheless, could hardly prevent some building up of recessive genes with harmful effects in a self-pollinating complex-heterozygous individual, and plants arising as homozygotes for one of its complexes through a loss of lethals would still, by and large, be at a selective disadvantage. This is evidenced by the fact that 7-paired segregates which have arisen from time to time in our garden from the races *grandiflora* and *suaveolens* have been systemically weakened and partially chlorotic. These theoretical considerations plus the radical phenotypic changes that would be required to transform alpha *biennis* 1 or alpha *biennis* 2 complexes into complexes of the *argillicola* type argue strongly against the possibility that plants in any of the present day *biennis* groups acted as progenitors of the argillicolas.

The similarities, however, between the end arrangements of the alphas of *biennis* groups 1 and 2 and the argillicolas probably do have phylogenetic meaning. In all probability they reflect the fact that none of these groups has evolved very far from the original ancestral population as far as end arrangement is concerned. Segmentally, the argillicolas are related to the alpha complexes of *biennis* groups 1 and 2 through the common ancestors, the hookeris or *hookeri*-like progenitors.

Other possibilities of origin

If the *biennis* groups do not apparently qualify for the role of progenitors of the argillicolas, what other possibilities can be suggested? In attempting to answer this question it is necessary to keep in mind the dual relationships of the argillicolas. The absence of balanced lethals, the presence of mainly paired chromosomes, of large, open-pollinated flowers and of segmental end arrangements which are close to the [b]*Johansen* arrangement very definitely link the argillicolas to the ancestral population in the far West. On the other hand, in their morphology, geographical distribution, habitat preference (in some cases at least), and influence on chlorophyll formation, the argillicolas resemble the beta *parviflora* complexes. In a way, they seem to occupy a position midway between the ancestral type and the parvifloras.

Considering first the resemblances between the *argillicola* population and the ancestral group, now most closely represented by the hookeris, there can be little doubt that the cytogenetic behavior and segmental arrangements which the two groups possess in common are indicative of a connection between them. As has been shown, it is difficult to suppose that the argillicolas have acquired their characteristics through loss of balanced lethals. Further, reversion to the original type of end arrangement is not probable (see below). Paired chromosomes, alethal complexes, open-pollination and ancestral end arrangements must be regarded as primitive characters derived from the ancestral population and never lost. The argillicolas are, therefore, interpreted as a relict population, the last remnants of a once more widely spread group which originally invaded the East from the Southwest.

The *argillicola* population may represent a case of great phenotypic divergence which has not been accompanied by extensive alteration in segmental arrangement, or else it may represent a population whose phenotypic characters are primitive in the subgenus Euoenothera. In evaluating these two alternatives it will be helpful to consider briefly the possible relationship between the argillicolas and another ancient eastern Euoenothera population, the grandifloras. Like the argillicolas, the *grandiflora* races are close to the *hookeri* group in segmental end arrangements and cytogenetic behavior but quite distinct phenotypically from the hookeris. STEINER (1952) has shown that there is reason to believe that the grandifloras are also relics of a formerly widespread population, which gave rise to the alpha complexes of *biennis* group 1. The question naturally arises as to whether the argillicolas and grandifloras are relics of the same population. Although this question cannot be answered with certainty at present, there are some indications that the argillicolas may be an older relict group than the grandifloras.

First, in their morphology the argillicolas are strikingly distinctive. Except for their resemblance to the beta parvifloras, they are far removed morphologically from all other euoenotheras. This great phenotypic divergence may indicate that the argillicolas were early separated from the rest of the subgenus, with the result that most traces of morphological relationship have been lost. Second, the fact that *argillicola* plastids differ not only from *hookeri* plastids, but from every other class of plastids yet discovered in the euoenotheras might be additional evidence that the argillicolas are a very old group. Finally, the sepal tip and stem tip characters of the argillicolas, although uncommon in the subgenus Euoenothera, are found in the subgenus Raimannia, as CLELAND (1949) has noted. It is barely possible that the early migration, of which the argillicolas are relics, may also have given rise to the progenitors of the raimannias. This also suggests that the argillicolas are an old group.

The original *argillicola* population may then have been the earliest Euoenothera assemblage to occupy eastern North America or even the first group to migrate from the ancestral home in the Southwest. It is conceivable, therefore, that the *argillicola* phenotypic characters may more closely approximate the primitive vegetative morphology of the subgenus than do the hookeris. Statements regarding the primitive morphology are dangerous, however, in the absence of fossil records. It would appear equally likely that as a result of their long isolation from the primitive group the argillicolas have become far removed from the original condition in morphological characters. In brief, then, the argillicolas are very likely an old eastern group, perhaps the oldest; but there can be no definitive statement relative to the primitive or derived nature of their phenotypic characters.

If the argillicolas are to be linked with the ancestral population of the Southwest, what significance is to be attached to their affinities to the beta parvifloras? Despite the similarities between the beta *parviflora* complexes and the argillicolas we do not think it likely that the beta *parviflora* complexes are ancestral to the argillicolas for the following reasons: (1) As a group the beta

parviflora complexes are segmentally the most diverse known, and are on the average the farthest removed from the original. In contrast to this, the *argillicola* complexes are relatively homogeneous in end arrangements and are close to the original. It is not probable that the beta *parviflora* complexes, which in the course of evolution have diverged in different directions from the original arrangement, would revert almost to the original segmental condition to give rise to the argillicolas. (2) The beta *parviflora* complexes possess lethals, and occur in races which bear circles of fourteen, whereas the majority of *argillicola* complexes are alethal and exist in structurally homozygous races. As in the case previously discussed, the chances of survival for a homozygous, alethal population which had been derived from a circle-bearing, permanently heterozygous population would probably be low.

The similarities between the two groups cannot be ignored, nevertheless, and phylogenetic relationships between them almost certainly exist. The interpretation of the argillicolas as a relic of a formerly more widely distributed population has, in all probability, significance for the evolution of the parvifloras. It seems entirely possible that the ancient *argillicola* population, which was very likely cytogenetically like the present day group, served as the progenitor of the beta *parviflora* complexes. As eastwardly moving *strigosa* and *biennis* groups invaded the domain of the argillicolas, they could well have hybridized with the latter, thus giving rise to the incipient *parviflora* populations. Then as the parvifloras and other groups evolved in the East, they may have replaced all but the now existing *argillicola* assemblage, which has succeeded in surviving because it has developed the ability to occupy a special ecological niche. This hypothesis, for which more evidence will be presented in another publication dealing with the parvifloras, would account for the similarities between the *argillicola* and beta *parviflora* complexes.

SUMMARY

Races of *Oe. argillicola,* a shale barren endemic of the Appalachian Mountains ranging from southwest Virginia to southern Pennsylvania, are unusual among eastern euoenotheras in their tendency to display in only minor degree the cytogenetic aberrations characteristic of the subgenus, as well as in their restriction to a special habitat. These *argillicola* forms show either all-paired chromosomes or small circles and several pairs at meiosis; they lack balanced lethal systems as a rule, and possess large open-pollinated flowers. Hybridization among members of natural populations was demonstrated in the group, which belongs to a subgenus where hybridization has been assumed to play a major role in the delimitation of phylogenetic units.

In segmental end arrangements the argillicolas show a close relationship with the putative ancestral population of the Southwest, represented at the present time by the hookeris. Thus, in the presence of mostly paired chromosomes, alethal complexes, open-pollination, and ancestral end arrangements the argillicolas are more akin to this group than to any eastern group.

Argillicola genomes were found to exert deleterious effects on the synthesis of chlorophyll in the plastids of certain races belonging to other phylogenetic groupings. Comparison of *argillicola* and beta *parviflora* complexes revealed that the two groups behave in a similar way in this respect. Both classes of complexes carry genic factors which, in combination with the genomes of other races, react with the plastids of these races in such a way that chlorophyll production in the plastids is blocked. On the other hand, *argillicola* and *parviflora* plastids are not identical in their reactions with given foreign genomes; *argillicola* plastids are incompatible with complex-combinations involving foreign genomes, whereas *parviflora* plastids are compatible with almost all genome combinations.

Morphologically, the argillicolas do not resemble the hookeris, but closely approach the beta parvifloras, in that both groups carry factors for narrow, thick, glabrous leaves, subterminal sepal tips, and bent stem tips. Another connection between these two groups is indicated by the fact that the parvifloras are able to occupy the shale barren habit of the argillicolas.

Regarding the phylogenetic position of the argillicolas, it is considered unlikely that they have been derived from any contiguous *biennis* or *parviflora* populations. The latter groups are rejected primarily on the grounds that they consist of circle-bearing, self-pollinating complex-heterozygotes with balanced lethal systems. It is not probable that such populations would, under natural conditions, give rise to 7-paired, lethal-free, open-pollinated populations, since there would apparently be strong selection pressure against evolution in this direction.

The occurrence of primitive characters in the *argillicola* group is interpreted to mean that it is a relict population of a once more widely distributed form, which invaded the East from the ancestral home in the Southwest. The possession of characters in common with the beta parvifloras is taken to indicate that the argillicolas have played a role in the origin of the parvifloras, probably by crossing with ancient *strigosa* and *biennis* populations in the East.

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THE EFFECT OF RADIATION DOSE FRACTIONATION ON CHROMOSOME ABERRATION FREQUENCIES IN TRADESCANTIA MICROSPORES. I. STUDIES WITH X-RAYS[1]

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THE role of time-intensity relationships in the production of chromosome aberrations by X-irradiation was first studied in Tradescantia by SAX (1939). By examination of microspores five days after X-irradiation, using a total dose of 320 r, he showed that the frequency of chromosome aberrations (dicentrics and centric rings) could be reduced 40% by increasing the duration of irradiation by a factor of 16. This intensity effect has long since become well established through the experimentation of SAX (1940, 1941, 1950) and a number of other investigators (CATCHESIDE *et al.* 1946a; MARINELLI *et al.* 1942). SAX also demonstrated (1939) that a reduction in 2-hit aberrations could be obtained by fractionating the total dose into a number of parts instead of giving it all at once and concluded (1940) that for 2-hit aberrations "most of the unions occur within a few minutes, and at the end of *about an hour* all fusions are complete. This does not necessarily mean, however, that a broken end can remain in an unstable condition for only an hour." LEA and CATCHESIDE (1942) upon a mathematical treatment of the data of SAX concluded that the average time between breakage and reunion is about 3½ minutes, although a few breaks may not restitute and may remain open for a longer period of time. Additional experiments of CATCHESIDE *et al.* (1946b) suggested that if restitution or reunion of newly broken ends is not accomplished within a few minutes, then it may be a long time, measured in hours, before more or less accidental movements bring the ends within the range of attraction again.

LANE (1951) in experiments "designed to obtain more critical information" on the effects of fractionation due to the "deficiencies in design and inference" in the work of previous investigators, confirmed the early experiments of SAX, when the interval between fractions is increased up to 4 hours. His experimental results indicated, however, that the aberration frequency rises again when the fractionation interval is increased to 6 or 8 hours and, furthermore, that at 4 hours' fractionation the frequency of aberrations drops considerably *below* that from twice the half dose. This apparent depressing effect on the sensitivity of chromosomes to breakage, he feels, is due to a temporary accumulation of nucleic acid on the resting stage chromosomes

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which reaches a maximum at about 4 hours after the first dose. The 1949 experiments of LANE were performed under controlled temperature and lighting conditions to reduce to a minimum the effect of uncontrolled environmental conditions on the rate of microsporogenesis during the period between irradiation and fixation. His experimental results have led him to draw a number of conclusions, among them, the rejection of the theory of rapid reunion and restitution, in favor of one where reunion and restitution occur more than 8 hours after breakage.

SAX and LUIPPOLD (1952), in an experiment designed to extend and complement the original observations of SAX, obtained results quite different from those of LANE. On the contention that environmental conditions such as temperature are effective, but limited to the period during and shortly after irradiation, the experiments were performed in their original form. Their results show the same reduction of 2-hit chromosome aberrations, with rest periods of 4, 8 or 12 hours between fractions. Further, this reduction does not exceed twice the 2-hit aberration frequency induced by a single half dose. In addition, the aberration frequency with 4, 8 or 12 hours fractionation is much less (40%) than that obtained from a full continuous dose.

LANE (1952) has objected to these results on the basis of uncontrolled environmental conditions, a difference in intensity levels used, a difference in material (*T. paludosa* versus *T. bracteata*), and a difference in the time period between irradiation and fixation.

Since the validity of a number of basic theories established over a period of years had been questioned so strongly and the evidence for rapid reunion and restitution had been rejected so thoroughly by LANE, it seemed desirable to design experiments using experimental methods similar to those of both SAX and LUIPPOLD (1952) and LANE (1951) to test the validity of the results obtained.

EXPERIMENTAL METHODS

Plan of experiments

The experiments were planned with a twofold purpose in mind: (1) to investigate the effect of fractionation at a low intensity using "uncontrolled" environmental conditions before and after X-irradiation in contrast to LANE's low intensity fractionation experiments with a "controlled" environment, and (2) to investigate the effect of fractionation at a high intensity using "controlled" environmental conditions in contrast to SAX and LUIPPOLD's high intensity fractionation experiment with "uncontrolled" environmental conditions before and after X-irradiation.

Materials and growth conditions

The plants used in both experiments were derivatives of clone 5 of *Tradescantia paludosa* (obtained from SAX) maintained at Yale University and at the Biology Division of the Brookhaven National Laboratory. The plants are ideal since they can be maintained in good flowering condition the year around in the greenhouse under standard greenhouse conditions. Inflorescences from

at least 40–50 different plants were used for each experiment to reduce to a minimum the effects one might obtain from intra- and interplant variation within the clone.

(1) In the low intensity experiment, young inflorescences were cut early in the morning of the day of the experiment and placed in the X-ray room prior to irradiation. After X-irradiation at 25°C (X-ray room temperature) the inflorescences were placed in the greenhouse where they remained until fixation.

(2) In the high intensity experiment, inflorescences were picked 12 hours prior to irradiation time, trimmed to a constant stem length, and were placed in a well-ventilated constant temperature room at 23–24°C under two different artificial lighting conditions.

Series A was placed at a distance of 9–11 inches from a light source of six 40-watt fluorescent bulbs and was given continuous light throughout the experiment.

Series B was placed at a distance of 6 feet from a light source of four 40-watt fluorescent bulbs and two 100-watt incandescent bulbs and was placed on an eight-hour photoperiod.

Irradiation conditions

(1) In the low intensity experiment (Yale University) the X-irradiation was administered at 250 KV and 6 mA, at a distance of 80.9 cm from the target, using a filter of 1 mm of aluminum. The dosage rate of 25 r/min was constant for each exposure and was checked with a dosimeter. The total dose was 350 r. During the course of the experiment, the temperature of the X-ray room varied from 25–26°C.

(2) In the high intensity experiment (Brookhaven National Laboratory) the X-irradiation was administered at 250 KV and 29 mA, at a distance of 46.0 cm from the target, using a filter of 1 mm of aluminum. The dosage rate of 350 r/min was constant and checked with a dosimeter after each exposure. The total dose in this experiment too was 350 r. Inflorescences of Series A and Series B representing either the full continuous dose, half-dose or fractionated doses, were irradiated simultaneously to insure uniformity of dose in comparative cases under the two lighting conditions.

Inflorescences were removed from the constant temperature room and transferred within 15 seconds to a constant temperature exposure chamber (23–23.5°C) for the X-irradiation. After irradiation they were put back into the constant temperature room under the appropriate lighting conditions until the time of the next fractionation, and subsequent slide preparation.

Slide preparation and analysis

At 72 and 96 hours after irradiation a portion of one of the anthers of a bud was tested, using smear techniques, with aceto-carmine for an abundance of metaphase cells. If the smear was satisfactory, the six anthers were smeared on a slide and fixed in fresh Carnoy's fixative. The slides were then stained as needed with propionic carmine and examined for aberrations using temporary mount preparations.

TABLE 1

The effect of fractionated dosages at low intensity on the aberration frequency per cell in Tradescantia paludosa microspores. May 1952.

Dose in roentgens	Fractionation time (hours)	No. cells	Interchanges per cell	Interstitial deletions per cell
350	0	694	0.48 ± 0.02	0.61 ± 0.03
175 + 175	1	568	0.43 ± 0.03	0.37 ± 0.03
175 + 175	2	600	0.35 ± 0.02	0.37 ± 0.03
175 + 175	4	462	0.32 ± 0.03	0.37 ± 0.03
175 + 175	8	679	0.41 ± 0.02	0.41 ± 0.03
175 + 175	12	937	0.36 ± 0.02	0.36 ± 0.02
175	0	690	0.17 ± 0.02	0.18 ± 0.02

In the analysis, late metaphase and anaphase chromosomes were scored. In all cases, no more than 100 cells per slide were scored, so each total count of 500 cells in tables 1 and 2 represents that accumulated from five different slides. In general, the agreement from slide to slide within each irradiation was very good, although in a few cases considerable variation from the mean was encountered. This again, stresses the importance of using inflorescences from a large number of plants in this type of work and scoring a large number of cells from a number of slides (SAX 1941). Standard errors have been calculated by the method discussed by CATCHESIDE *et al.* (1946a).

Cells with abnormal chromosome numbers (diploid, monosomics or trisomics) were rejected in the analysis as well as those cells, which occurred very rarely, in which chromatid aberration types appeared.

In tables 1 and 2, interchanges refer to dicentric, tricentric (scored as two interchanges) and centric rings, and interstitial deletions refer to acentric fragments not associated with interchanges. Difficulties in the analysis of acentric

TABLE 2

The effect of fractionated dosages at high intensity on the aberration frequency per cell in Tradescantia paludosa microspores as modified by different lighting conditions before and after irradiation. Temperature throughout the experiment 24°C. August 1952.

Series	Dose in roentgens	Fractionation time (hours)	No. cells	Interchanges per cell	Interstitial deletions per cell
A[1]	350	0	500	0.54 ± 0.03	0.85 ± 0.04
A	175 + 175	1	600	0.38 ± 0.03	0.55 ± 0.03
A	175 + 175	2	500	0.35 ± 0.03	0.53 ± 0.03
A	175 + 175	4	500	0.34 ± 0.03	0.53 ± 0.03
A	175 + 175	8	500	0.29 ± 0.02	0.54 ± 0.03
A	175 + 175	12	500	0.31 ± 0.03	0.47 ± 0.03
A	175	0	500	0.16 ± 0.02	0.25 ± 0.02
B[2]	350	0	500	0.63 ± 0.04	0.84 ± 0.04
B	175 + 175	4	500	0.41 ± 0.03	0.54 ± 0.04
B	175 + 175	12	500	0.35 ± 0.03	0.51 ± 0.03
B	175	0	500	0.19 ± 0.02	0.25 ± 0.02

[1] Continuous light.
[2] 8-hour photoperiod.

fragments, as has been well illustrated by LANE (1951), were overcome by classifying as deletions all pairs of acentric fragments in excess of one per dicentric chromosome or centric ring, or two per tricentric chromosome. Thus, deletions in this method of analysis include fragments of 1-hit and 2-hit origin (RICK 1940).

EXPERIMENTAL RESULTS

In both the low and high intensity experiments, a total dose of 350 r was administered. A continuous dose of 350 r and a single half-dose of 175 r were given for comparison with the fractionated doses separated by intervals of 1, 2, 4, 8 and 12 hours.

The results of the low intensity experiment are summarized in table 1 and figure 1. The reduction in interchange frequency at 25 r/min as a result of 4 to 12 hours of fractionation is about 30% that of the full continuous dose and the reduction in deletion frequency is about 27% that of the full continuous dose. In either case, the aberration frequency reduction at 4, 8 or 12 hours never falls below twice the aberration frequency induced by a single exposure of 175 r (base line of SAX's curves).

The results of the high intensity experiment are summarized in table 2 and figures 2 and 3. The reduction in interchange and deletion frequency under both lighting conditions, at a constant temperature of 24°C throughout the experiment, as a result of 4, 8 or 12 hours of fractionation is about 40% that of the full continuous dose.

Whereas the deletion frequency curves in figures 2 and 3 coincide for both lighting conditions, it can be seen that the interchange frequency curves, although identical in shape, show a uniformly higher interchange frequency for those inflorescences maintained under an eight-hour photoperiod than those kept under continuous light.

The fundamental relationship, however, of the interchange frequencies at 4, 8 and 12 hours of fractionation not exceeding that of twice the aberration frequency produced by a single dose of 175 r has not been altered by the different environmental conditions. Whether light bears a relationship to total interchange frequency, as seems possible in these experiments, will have to be determined more accurately in further experimentation designed expressly for that purpose. Preliminary experiments, to date, along these lines suggest, however, that this relationship may be due, rather, to any one of a number of personal factors in experimentation methods or scoring.

DISCUSSION

As stated previously, the experiments presented in this paper were designed to act as controls on both the experiments of SAX and LUIPPOLD and those of LANE. What we have considered LANE's most valid objection, that of "uncontrolled" environmental conditions after irradiation, has been shown in these experiments to have no effect on the 2-hit aberration frequencies obtained after 4-, 8- or 12-hour intervals between fractions.

In the low intensity experiment (25 r/min) no particular precaution was taken after X-irradiation (as in SAX's experiments) and the inflorescences were left in the greenhouse until fixation. The temperature was, of course, "controlled" during the irradiation and varied from 24 to 26°C. Nevertheless, the fundamental relationships as shown by SAX and LUIPPOLD (1952) are the same in this low intensity experiment. In no instance did we find that

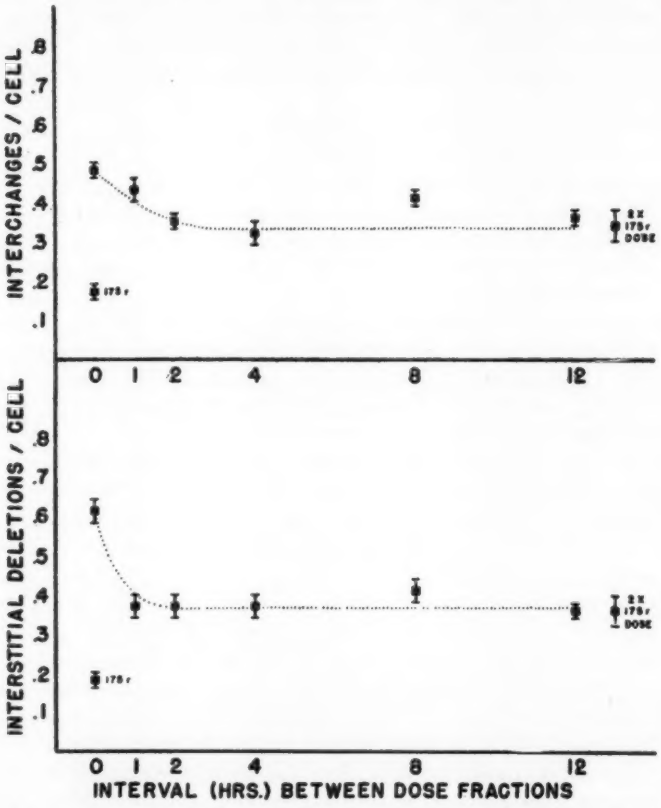

FIGURE 1.—Interchange and interstitial deletion frequencies per cell with a dose of 350 r at an intensity of .25 r/min.

at 4, 8 or 12 hours fractionation, the 2-hit aberration frequency fell significantly below twice the 2-hit aberration frequency of a single half-dose. At no point from 4 to 12 hours of fractionation (as proposed by LANE) did we find evidence for a significant increase in aberration frequency due to a "recovery" in sensitivity to breakage.

However, even though this experiment was performed at the same intensity

and buds were fixed at the same or equivalent time after irradiation, objections might still be made on the grounds of uncontrolled environmental conditions after irradiation.

The high intensity experiment was performed in a manner closely allied to the 1949 low intensity experiments of LANE (1951). Temperature was controlled before, during and after irradiation with a maximum variation of 23–

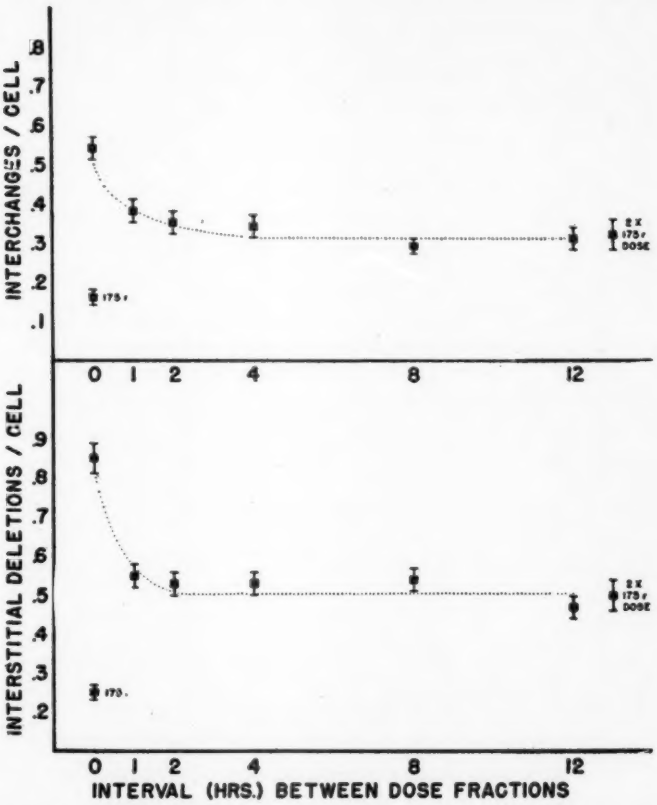

FIGURE 2.—Interchange and interstitial deletion frequencies per cell with a dose of 350 r at an intensity of 350 r/min. (continuous light).

24°C. The lighting conditions were duplicated and buds were kept in continuous light throughout the experiment (series A). Buds were again fixed at equivalent times after irradiation.

An examination of the interchange frequency curves in figures 2 and 3 shows that, even where temperature has been held constant, with variable lighting conditions, the results obtained at 4, 8 and 12 hours of fractionation

are again the same as those found in the low intensity experiment. The 2-hit aberration frequencies from 4, 8 or 12 hours of fractionation do not differ significantly from twice the 2-hit aberration frequency obtained from a single half-dose. Thus, even the use of LANE's exacting experimental method does not yield experimental results in accordance with those resulting from his 1949 experimentation.

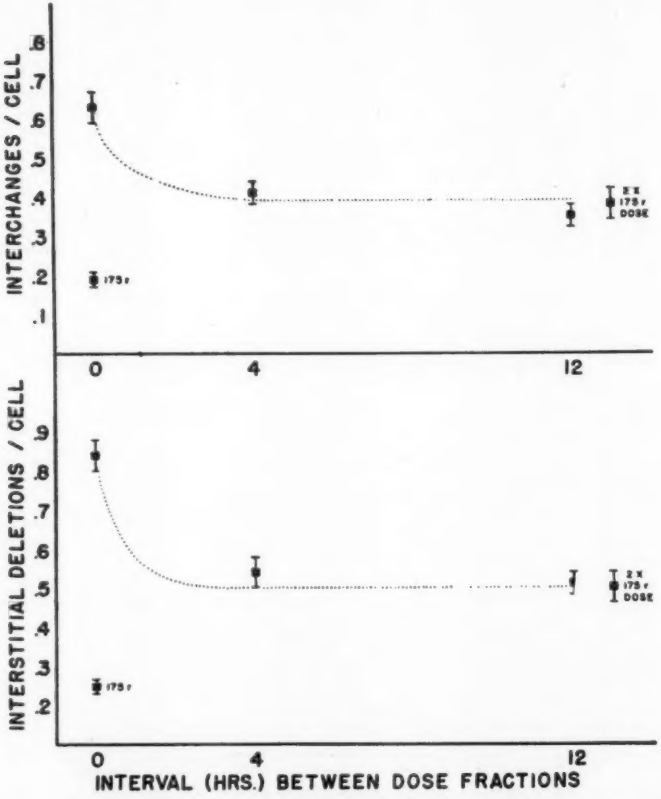

FIGURE 3.—Interchange and interstitial deletion frequencies per cell with a dose of 350 r at an intensity of 350 r/min. (8-hour photoperiod).

We, then, are forced to the same conclusions as SAX and LUIPPOLD (1952): that the differences in experimental results are due to inadequate data as a result of the low numbers of cells (chromosomes) scored by LANE. Considerable variation in microspores from anthers which have apparently received the same treatment has been encountered previously by a number of investigators (SAX 1941; CATCHESIDE, LEA and THODAY 1946a).

SUMMARY

Experiments have been performed at a low and a high intensity of X-rays on Tradescantia microspores to test the validity of the theory, recently proposed by LANE, that there is an inhibition-recovery sequence in the production of chromosome aberrations with fractionated doses. In both experiments single doses of 350 r and 175 r were administered without fractionation. In addition, five exposures of 350 r were administered in two equal parts with intervals of either 1, 2, 4, 8 or 12 hours between fractions. The results of "controlled" environmental conditions (light and temperature) in the high intensity experiment and "uncontrolled" environmental conditions in the low intensity experiment do not support the inhibition-recovery sequence theory. The results of both experiments support, rather, the original and later conclusions of SAX that the reduction in chromosome aberration frequency with rest periods of 4, 8 or 12 hours between doses does not exceed twice the aberration frequency induced by a single half-dose. In none of the intervals tested did the aberration frequency drop significantly below that induced by twice a single half-dose. Further, the experimental data provide no evidence for a significant increase in aberration frequencies with 4- to 12-hour intervals between fractions, due to a "recovery" in sensitivity to breakage, as proposed by LANE.

ACKNOWLEDGMENT

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THE EFFECT OF RADIATION DOSE FRACTIONATION ON CHROMOSOME ABERRATION FREQUENCIES IN TRADESCANTIA MICROSPORES. II. STUDIES WITH FAST NEUTRONS[1]

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THE previous paper (DE SERRES and GILES 1953) has described the results of experiments investigating the effects of fractionated doses of X-rays on the frequency of chromosome aberrations induced in Tradescantia microspores. The results of these studies demonstrate that there is a marked effect of X-ray dosage fractionation in reducing the yield of 2-hit aberration types and, further, that no recovery from this reduced yield occurs when the period between dose fractions is greater than four hours. These conclusions are thus in agreement with those obtained in earlier experiments of SAX (1939, 1940) and more recent experiments of SAX and LUIPPOLD (1952), and do not agree with the observations of LANE (1951).

In the discussion and interpretation of his experimental results with X-rays, LANE also discussed some of the results of fast neutrons experiments. These experiments (GILES 1940, 1943; THODAY 1942) indicated that with fast neutrons, in contrast to the situation with X-rays, the relationship between radiation dose and interchange frequency is linear, suggesting that both breaks are the result of a single hit, both being produced by a single proton path. This interpretation was confirmed by experiments in which it was shown that there was no effect of the intensity of neutron radiation on either chromosome or chromatid interchange yields, and also that fractionation of a neutron dose had no effect on the frequency of chromatid interchanges. Since the intensity and dose fractionation results with neutrons are difficult to reconcile with LANE's interpretations of his X-ray results, LANE questions the validity of these results, although he presents no new data based on neutron experiments. Consequently, when experiments were being planned to reinvestigate the effects of fractionated doses of X-rays, it seemed desirable to perform additional complementary studies with neutrons. In the original experiments, most of the observations dealt with chromatid rather than chromosome effects and the time intervals between dose fractions did not exceed 32 minutes. It thus seemed desirable to determine experimentally whether fractionation would have an

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effect on the frequency of chromosome exchanges, particularly with longer time intervals between fractions. Experiments of this type are now much easier to perform, since more adequate facilities are available compared to the situation which obtained in the early experiments using cyclotron-produced neutrons.

MATERIALS AND METHODS

The same clone of *Tradescantia paludosa* (SAX No. 5) was used as in the X-ray experiments (DE SERRES and GILES 1953), and in general the procedures for slide preparation and analysis were similar. Inflorescences were irradiated with fast neutrons arising from uranium fission in a special exposure device in the Oak Ridge nuclear reactor as utilized and described in previous studies (GILES and CONGER 1950; GILES, BEATTY and RILEY 1952). All irradiations were performed at approximately 23.5°C in air. The inflorescences were kept at this temperature between dose fractions and were then removed to the greenhouse where they remained until slides were prepared at 72 and 96 hours after the end of the second radiation dose. The fast neutron dose was monitored with the same Victoreen thimble ionization chamber used in the experiments of GILES, BEATTY and RILEY (1952). The neutron intensity was approximately 2.5 N/min. Fractionated doses were administered in two five-minute exposures (12.5 N each) with intervals of 1, 2, 4, 8 and 12 hours between exposures. In addition, individual separate exposures of 5 minutes (12.5 N) and 10 minutes (25 N) without fractionation were made.

RESULTS AND DISCUSSION

The results of the analyses for frequencies of interchanges and of interstitial deletions arising from the several different exposures to fast neutrons are presented in table 1. In addition, these results are represented graphically in figure 1.

It is immediately clear that, in marked contrast with the X-ray results, there is no decrease in the frequencies of either aberration type with dose fractionation intervals up to 12 hours. Further, the values obtained by multiplying by

TABLE 1

Effect of fast neutron dose fractionation on the frequencies of chromosome interchanges and interstitial deletions induced in Tradescantia microspores.

Dose (N units)	Time interval (hrs.) between fractions	No. cells	Interchanges per cell	Interstitial deletions per cell
25	0	300	0.82 ± 0.05	0.80 ± 0.05
12.5 + 12.5	1	300	0.90 ± 0.05	0.90 ± 0.06
12.5 + 12.5	2	326	0.84 ± 0.06	0.90 ± 0.05
12.5 + 12.5	4	350	0.76 ± 0.05	0.90 ± 0.05
12.5 + 12.5	8	523	0.84 ± 0.04	0.83 ± 0.04
12.5 + 12.5	12	550	0.81 ± 0.04	0.92 ± 0.04
12.5	0	678	0.44 ± 0.03	0.44 ± 0.03

two the aberration frequencies arising from a single dose delivered in one five-minute exposure, agree well, as shown in figure 1, with those obtained from the ten-minute exposures, whether these were made in a single exposure or in two fractions.

The absence of an effect of dose fractionation with fast neutrons observed in these experiments on chromosome aberration types is in agreement with the

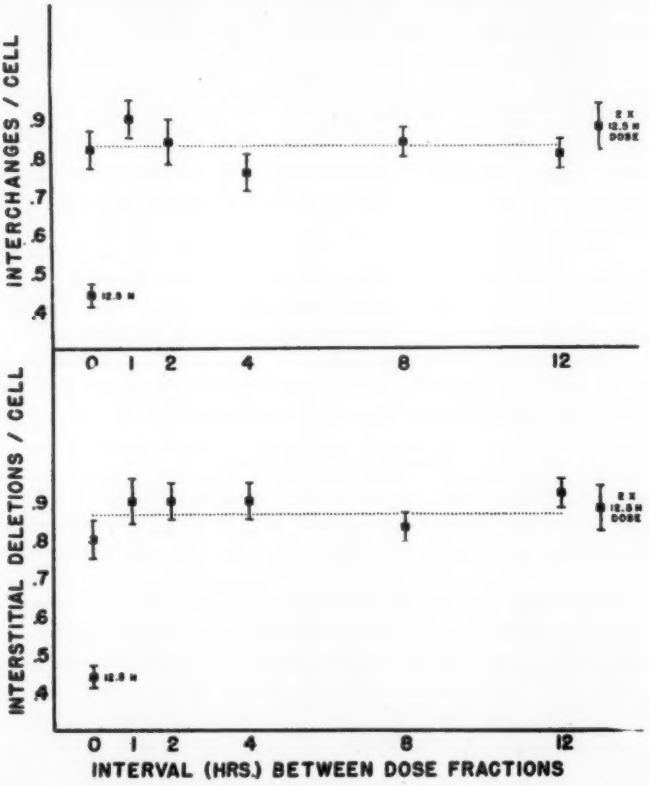

FIGURE 1.—Interchange and interstitial deletion frequencies per cell with a dose of 25 N at an intensity of 2.5 N/min.

results obtained in previous studies (GILES 1943) involving chromatid aberration types. These data do not support the contention of LANE (1951) that there exists an inhibition-recovery sequence in radiation experiments on chromosome breakage. The contrasting effects obtained in these experiments with neutrons and comparable ones with X-rays (DE SERRES and GILES 1953) support the original hypotheses of SAX (1939, 1940) and GILES (1940, 1943) as

to the mechanisms of X-ray and neutron-induced chromosome interchanges in Tradescantia microspores. With neutrons both breaks are still interpreted as arising simultaneously from a single event—the passage of a single recoil proton—and hence no effect of intensity or of dose fractionation is anticipated. With X-rays, however, the two breaks are interpreted as arising independently as a result of the passage of separate secondary electrons. The reduction in yield of interchanges with lowered intensities or with fractionated doses is still most adequately explained on the view that relatively rapid restitution occurs following breakage.

SUMMARY

Experiments have been performed with fast neutrons to test the effect of dose fractionation on the frequencies of chromosome aberrations (interchanges and interstitial deletions) induced in Tradescantia microspores. A single dose of 25 N was administered without fractionation, and in two equal fractions with intervals of 1, 2, 4, 8 and 12 hours between fractions. No effect of fractionation has been found; the frequencies of both types of aberrations studied were essentially the same for all treatments. Further, aberration frequencies obtained from a single dose of 12.5 N were approximately half those produced by 25 N. These results are in marked contrast to those obtained in similar comparative experiments with X-rays where a pronounced decrease in aberration frequencies, with no recovery even with intervals between fractions as long as 12 hours, results from dose fractionation. The data obtained in the present experiments with fast neutrons are in agreement with previous hypotheses as to the mechanisms by which radiation-induced chromosome aberrations arise in Tradescantia. They do not support the contention that there exists an inhibition-recovery sequence in the production by radiation of chromosome breakage.

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